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CONTENTS

	PAGE
METABOLISM IN PREGNANCY. *Victor John Harding*	279
BLOOD PRESSURES IN MAN UNDER NORMAL AND PATHOLOGICAL CONDITIONS. *J. A. MacWilliam*	303
PERVERTED APPETITES. *Henry H. Green*	336
THE PHYSICAL CHEMISTRY OF THE PROTEINS. *Edwin J. Cohn*	349

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PHYSIOLOGICAL REVIEWS

VOL. V JULY, 1925 No. 3

METABOLISM IN PREGNANCY

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As by metabolism is meant the general exchange of energy and material occurring in a mass of living cells, any review of the subject of metabolism in pregnancy should be an attempt to show how the metabolism of the adult, which is in a condition of equilibrium, is affected by the superposition of the metabolism of the growing fetus, in which anabolic reactions predominate. How are the demands for necessary building material and energy met by the maternal organism, and in meeting those demands how far, if at all, are the various parts of the metabolic equilibrium existing in the mother altered? This question has been profoundly influenced in the case of the human organism by the various views which have been held from time to time on the nature of the "toxemias of pregnancy." As in so many other instances where the possibility of clinical application has occurred, the pathological has been studied, almost to the exclusion of the physiological, certainly without adequate knowledge of the variations of the latter. In this review attention will be paid chiefly to human pregnancy.

THE GROWTH OF THE FETUS AND MATERNAL ORGANS. The prenatal growth of the human body was early a subject of study by pathologists and anatomists. This early work is summarised by Jackson (1). Relatively, growth proceeds with extreme rapidity in the first lunar month of intrauterine life, gradually to decline, until at birth it is only a minute fraction of its early value. Thus the rates are as 9999 to 0.45 comparing the first and last lunar months. Keene and Hewer (2) have suggested, however, that most probably the relative rate does not decrease evenly during pregnancy, but suffers a fairly marked diminution from about the seventh to the ninth lunar month, increasing in the terminal period. This they make evident not only from their own figures, which are rather few in number for this purpose, but also from the fetal growth curves of Donaldson.

Although the relative growth rate of the fetus may diminish at the eighth month, its absolute weight continues to increase; indeed, at about this time it is growing at the rate of from 500 to 800 grams per

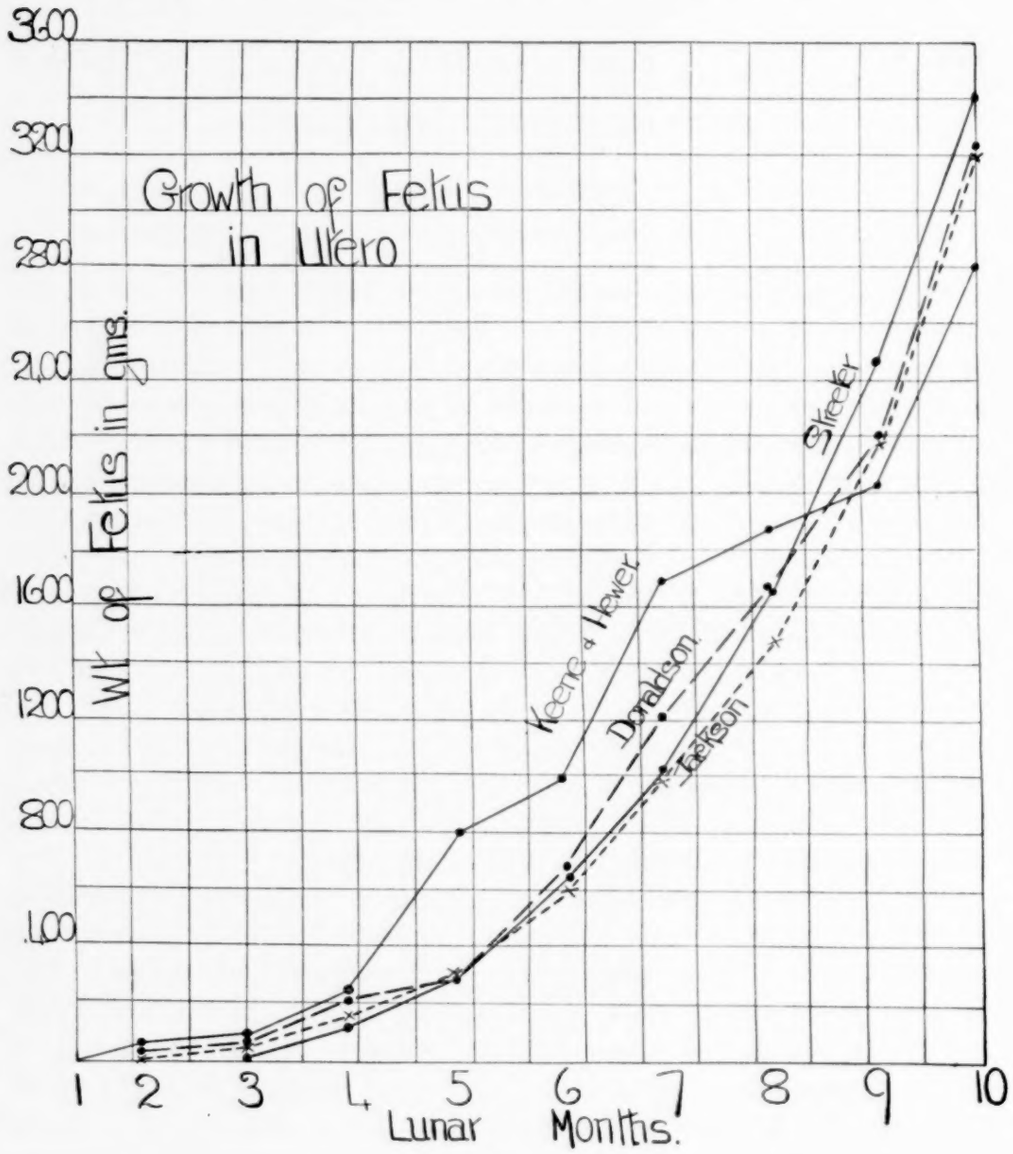

Fig. 1

month. In figure 1 are given the growth curves in utero of the human fetus from the data of Jackson, Donaldson (3), Streeter (4) and Keene and Hewer. (The figures of Streeter include a series by Zangemeister (5).) It will be seen from the curves that up to the fifth month the

weight of the fetus forms only an inconsequential fraction of the total maternal mass, and that even at the sixth month it forms hardly more than 1 per cent of the whole weight of a 60 kgm. woman. From that point, however, it increases rapidly until at term it constitutes 5 to 7 per cent of the original weight of the mother. In addition the hypertrophy of the mammae, and fetal adnexa constitute a further gain of some 3 kgm. of active tissue growth. These changes again are most marked in the later months of pregnancy. Viewed from the standpoint of mass alone pregnancy could thus exert little influence on either the energy or material exchange until about the sixth month. Any influence it may have before that time must be due to some cause inherent in the state of pregnancy itself.

The growth curves of the fetal organs follow much the same course as the total growth. Keene and Hewer find an actual diminution in growth in the thyroid, thymus, liver, spleen, super-renals and kidneys at about the eighth month.

The composition of the fetus during its uterine growth was studied in the earlier work of Fehling (6), Michel (7) and Hugounenq (8), and their figures have been made the basis of studies of the nitrogen and mineral balance during pregnancy. Indeed, on the grosser composition of the fetus no other figures seem available. More recent values on the Ca content are to be found in the work of Givens and Macy (9) and Schmitz (10), whose papers should be consulted for the details. Givens and Macy also paid attention to the amount of Mg. Shohl (11) in a recent review, has discussed the composition of the ash of the new-born. In figure 2 there have been collected the main results, and the relation of the composition of the fetus to general growth.

It may be noted, however, that there are many gaps in our knowledge of the composition of the developing human muscle, particularly in the region of that class of substances known generally as extractives. The occurrence of these substances has been better studied in other developing organisms than mammals, though even here knowledge is scanty. Wells and Corper (12) isolated xanthine and hypoxanthine from autolysed full-term fetal tissue and noted the presence of guanase and adenase in early fetuses occurring before the development of xanthooxidase.

The most prominent changes in the maternal organism are naturally those of the enlarging uterus. From a relatively inert organ of about 30 grams it becomes a powerfully contracting muscle of about 1 kgm. in the space of 40 weeks, only to be reduced to its original weight and

state in an even less space of time. The greater part of its growth, however, is in the first three months of pregnancy.

The placenta, acting as it does, as a medium of interchange of products of maternal and fetal blood, has been the subject of much more attention. The work on the grosser chemical composition is ably reviewed in an article by Koelker and Slemons (13) in 1911, and some of the main

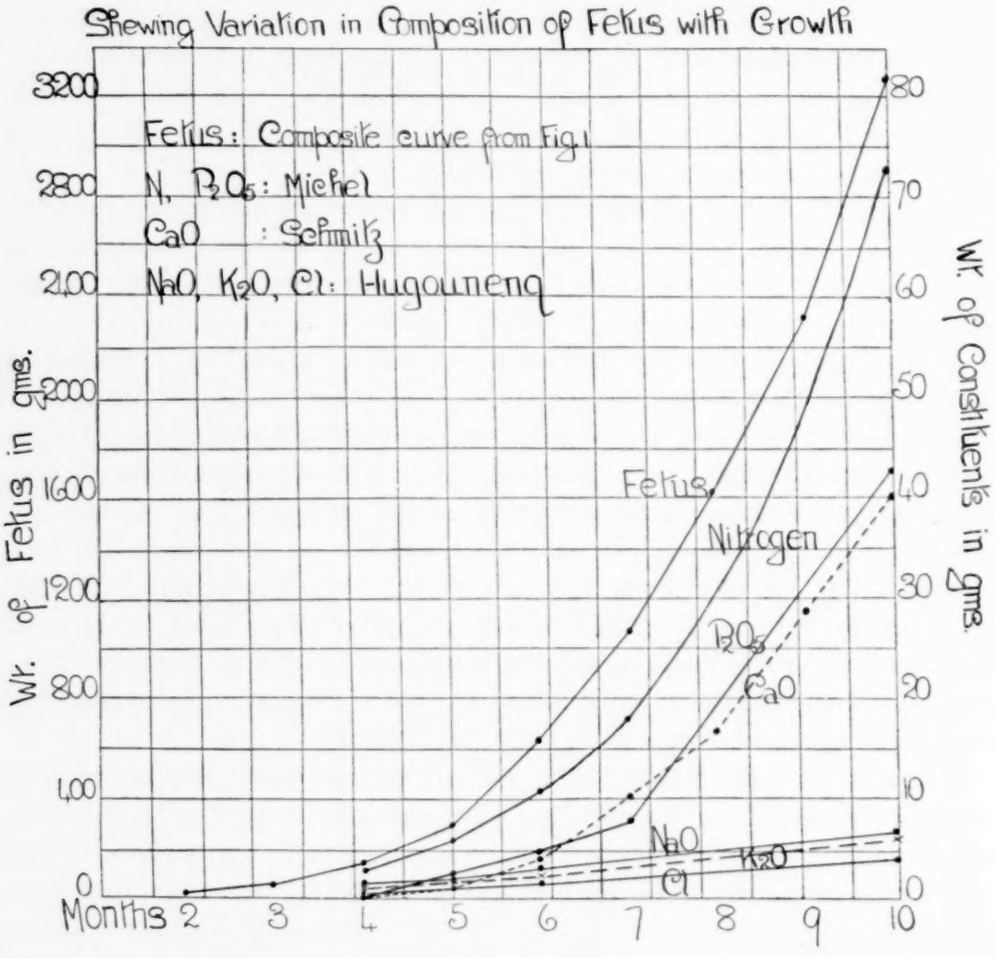

Fig. 2

points were confirmed in 1918 by Harding and Fort (14). These latter, however, obtained twice as much arginine in placenta proteins as obtained in a similar way from other human tissues. The significance of this is not clear. Glycogen is of constant occurrence in the placenta, occurring to the extent of from 0.49 to 0.58 per cent of the gross weight at term. Driessen (15), Flesch (16) and Todyo (17) have independently

shown that it is most abundant in the early part of pregnancy. Wells and Corper have shown the presence of hypoxanthine, adenenine and guanine. The presence of xanthine is doubtful. A most interesting observation is that of Fenger (18) on the composition of the placenta of the cow. In that animal the placenta occurs as a series of cotyledon-like masses which can be easily separated into maternal and fetal parts. The maternal parts maintain approximately the same composition in Cl, K, Na, Fe, Ca and Mg, independent of the period of pregnancy. The fetal parts, however, contain much more Cl, Ca and Na in the early part of pregnancy than at term. On the other hand they contain relatively much less iron in the early period. The fetal placenta in its content of Cl and Na would resemble that of the early fetus which, as seen from figure 2, contains relatively more of these elements at that period than at term. The figures probably represent those of the plasma bathing the cells. An increase in Ca would hardly be expected, as the increase in that element observed in the fetus is due to bone formation. The so-called "calcareous placenta" might, however, be of interest in this connection.

In addition to the gain in the uterus and the placenta the mother often experiences a general gain in tissue, consisting of new protein reserves and of fat deposited not only in the muscles of the pelvic region, but also being of general distribution throughout the body.

Changes have also been attributed to organs in the maternal body other than those immediately connected with the fetus or its nutrition. Thus the literature contains many references to the hypertrophy of the thyroid which has been recognised clinically in women. A long bibliography on this subject is given by Fruhinsholz and Parisot (19). Hypertrophy too has been noted in the parathyroids and failure to increase in size and activity during pregnancy has been given as the etiology of occasional cases of tetany. There can be little doubt that the anterior lobe of the pituitary gland undergoes marked increase in size during pregnancy, returning to its original condition after delivery. A recent review on this subject is given by Perez (20), but references can be found in most text-books on obstetrics or on internal secretions. The reason for this cycle of change is not clear, although Wallis and Bose (21) have suggested that "pregnant glycosuria" is connected with its excessive activity. The suprarenals are also known to undergo hypertrophy during pregnancy. Herring (22), using the rat as his experimental animal, noted very little difference in the weights of the heart, kidney and spleen; the liver and suprarenals were increased in weight,

while, contrary to expectations, the thyroid and pituitary were decreased. The thymus was also reduced as a result of pregnancy.

Kemper (23) has recently made an elaborate study of the total weight changes in human pregnancy, labor and the puerperal period.

NITROGEN METABOLISM. A full-grown human fetus contains no more than 100 grams N. In Michel's series of fetuses there was only a little over 70 grams N at term. If the curve of N growth of the fetus be plotted alongside the curve of general growth (as in fig. 2) there will be noticed a very clear general resemblance. The fetus lays down N very much in accordance with its general growth and preserves a fairly constant percentage of N. Yet other characters of the fetus alter markedly during growth. In its early growth period it contains large amounts of water which diminish later in intra-uterine life. Up to the middle of the seventh month it contains very little subcutaneous or visible fat. This component of the body is only laid down in large quantities in the later months of pregnancy (24).

Although protein is thus laid down much more evenly than fat in the fetus, yet the greatest increases occur in the later months. The way in which these N gains are made has been a subject of much early experimentation and many observations on animals other than man have been made in this connection. Much of the earlier work of Hagemann (25), Ver Ecke (26) and Jagerrooes (27) may be discounted on account of faulty technique. It led to the position that pregnancy inevitably meant a loss to the maternal organism whatever the diet. That this position is untenable was shown by the beautiful work of Bar (28) and Murlin (29) on the dog, and Hoffstrom (30), Landsberg (31) and Wilson (32) on woman. Other researches in this connection are those of Gammeltoft (33) on the goat, and Schrader (34), Sillevis (35), Slemons (36) and Hahl (37) on woman. There can be no doubt that given an adequate diet N gains are easily made in the latter half of pregnancy, whatever be the subject. Thus Bar and Murlin's experiments showed positive N balances in the dog on a daily intake of $\frac{2}{3}$ to $\frac{3}{4}$ gram N per kgm. body weight, and a corresponding calory intake of 70, in Murlin's experiments, and 80 to 100, in those of Bar. Hoffstrom found in women an average daily retention of 1.84 grams N, and Wilson's subjects, on N intakes varying from 9 to 19 grams N daily, showed positive N balances up to 6 grams. The calories in these latter cases varied from 35 to 50 per kgm. body weight. It is evident that N retentions of such magnitude are not only ample for the growth of the fetus and appendages, but must result in a gain to the mother herself. This has been

calculated in Hoffstrom's case and two of Wilson's to be 209, 284.5 and 210.9 grams N respectively. This laying down of N was supposed to act, both by Hoffstrom and Wilson, as a reserve store of protein material to provide for the needs of lactation. Certainly its amount is such as to give an ample yield of milk were it converted entirely into the proteins of that fluid during the post-partum period. Hoffstrom's figures on P retention during pregnancy show that this element also undergoes storage in the maternal organism; a fact which lends color to the hypothesis just suggested. Yet the hypothesis has not been put to any crucial test. Such retention would resemble to some extent the retention observed in many animals, including man, after periods of forced protein feeding and known as deposit protein. Moreover, the immediate puerperal period is marked by a N loss, a loss supposed to arise in part from the involuting uterus, yet N from this source is, at any rate, not wholly available for lactation purposes.

Although large N gains can thus be easily made in pregnancy, yet in the rabbit, dog and goat, such gains are apparently confined to the latter half. The first portion is marked by N losses. Even when the diet is adequate in N and calories to ensure equilibrium in the nonpregnant condition, and positive balances in the latter portion of a gestation, these N losses are observed. Gammeltoft even went so far as to state them to be inevitable, whatever the diet. In the early cases of woman observed by Landsberg and Wilson, N losses were not observed, though the positive balances were small, and not to be compared with those observed later in the pregnancy. This would appear to differentiate human pregnancy from that of other animals, but it must be remembered that the human fetus in its early stages of growth bears a very small proportion indeed to the total maternal mass. The smallest losses observed by Murlin in his experiments on the dog occurred when only one puppy was born as a result of the pregnancy. Mere mass of the fetus compared with that of the mother may thus be a deciding factor in the results. On the other hand, Crowther and Woodman (38) have made the observation of a similar loss of N on the dairy cow in early pregnancy, so that woman would appear to occupy an exceptional position in this regard. An explanation of this phenomenon was given by Murlin who regarded it as an effect of the parasitic action of the developing ovum on the uterine wall. The ovum in its early stages undoubtedly possesses a powerful digestive action. Proteolytic enzymes have been described in the two-months human ovum (39), and undoubtedly they occur at the earliest recognisable age. Teacher (40)

in his latest description of the early human ovum upholds this idea. The N losses which occur, however, are more than can be ascribed to merely local breakdown of the uterine wall by such a process of proteolysis. They must be much more general in character. They have thus been ascribed to the spread of such enzymes throughout the maternal cells, or to the liberation of products of proteolysis of the uterus into the maternal blood stream, thus causing a general protein breakdown. The explanation of this early negative N balance is a matter of some importance, as involved with it has been an explanation of the so-called physiological vomiting of women in early pregnancy. Both Bar and Murlin observed vomiting in some of their dogs. It is true that the N losses in the dog cease at about the time of the formation of the placenta in that animal, and it is well known that the nausea and vomiting of pregnancy in women often spontaneously disappears at a corresponding time in human gestation. Placenta formation would appear to be involved in the stoppage of both. The N losses which occur, however, in vomiting of pregnancy in woman are not inevitable in character as would be expected were they brought about by foreign enzymes, or an intoxication of the nature of a proteose intoxication. Harding and Potter (41) found that small amounts of carbohydrate administered in cases of vomiting of pregnancy in women led to a diminution of N output very much as in starvation, and Harding and Drew (42) found in the blood no other evidence than that of either starvation or dehydration, both of which could arise as a result of the condition. The subject, however, is well worth further study, especially in an animal such as the dog where the N losses can be easily observed.

Lauter (43) has made a study in four cases at the ninth month of the "wear and tear" quota of N in pregnancy. On liberal diets containing from 0.36 to 0.98 gram N he finds the urine N to be from 2.03 to 2.87 grams. Including the fecal N the total excretion was 2.975 to 3.99 grams; figures which compare favorably with the extremely painstaking work of Martin and Robison (44) on two normal men.

One cannot help but be struck by the economy with which the pregnant organism utilises valuable protein, and it is this economy, visible in the latter part of pregnancy, which renders the N losses occurring early in gestation in animals other than man, at once so inexplicable and so interesting.

Energy exchange. There can be little doubt that the addition of a mass of new and active protoplasmic tissue such as is represented by the fetus will result in an increase in the total metabolism of the preg-

nant organism as contrasted with the same organism in a non-pregnant condition. The new growth representing an addition of some 10 to 14 per cent of the original body weight must add an extra quota to the number of calories required for maintenance. The question which will naturally arise is—to what *extent* will it influence the energy metabolism? Will it act as so much extra maternal tissue, and the mother and fetus behave as a similar non-pregnant female but of a slightly increased weight and size; or, will the mother and fetus behave as two independent organisms and the total calories be the sum of the calories required by the mother in her non-pregnant condition plus the calories required by the fetus; or as a third possibility, is there something inherent in pregnancy itself which may affect the energy exchange in some incalculable manner? Reliable work on this subject commences with that of Magnus-Levy (45). He made observations on the oxygen consumption before conception and occasional determinations during pregnancy commencing at the third month. He noted a continual rise throughout pregnancy commencing even with the earliest observations. No one else has found an increase in metabolism at so early a period of pregnancy. All the other experiments show no rise until the sixth or seventh month of gestation. It is at this point, as is shown in a previous section of this review, that the mass of the fetus begins to be appreciable compared with that of the mother. Zuntz (46) confirmed Magnus-Levy in finding that later in pregnancy there was an undoubted rise in the total energy exchange. Murlin (47) in the same year made the important observation on the dog that the "extra energy production of the pregnancy at or near its culmination is proportional to the weight of offspring to be delivered." He was able to do this by observing a female bull terrier in two successive pregnancies, in which one and five puppies were produced respectively. Hasselbach (48) also noted an increased metabolism just before delivery as compared with the same woman one month later. Baer (49) in 1921 and Cornell (50) in 1922 made a series of single observations on a large number of cases at or near term, and noted, many times, large increases in basal metabolic rate in pregnancy compared with the usual non-pregnant standards. Their work, however, loses in value as many of the cases were not normal, and little account was taken of possible psychological factors. Wilson and Bourne (51), and Haselhurst and Plant (52) have also made a recent series of observations. One of the most carefully controlled pieces of work in this subject is that of Root and Root (53) who followed fortnightly the basal metabolic rate in a pregnant woman from the fourth

month until the ninth week of lactation. From the sixth month the rate steadily rose until some six weeks before delivery it was 23 per cent greater than at four months. The total increase in weight was but 14 per cent, and a non-pregnant woman showing a similar increase in weight would only have increased her metabolic rate 5 per cent according to either the tables of Harris and Benedict, or Aub and Du Bois. Similar results have been shown by Sandiford and Wheeler (54) in a well-controlled case, and Rowe, Alcott and Mortimer (55) in a series of human pregnancies.

Carpenter and Murlin (56) in 1911 compared the energy exchange of the pregnant woman and fetus in the ninth month with that of the mother and child post-partum in 3 cases. The metabolism was found to be the same to plus 4 per cent in favor of the pregnant woman. This would mean that there is "no deflection in the energy consumption curve at birth," and that the maternal organism and fetus function as two separate units in their consumption of energy. Sandiford and Wheeler have recently strengthened this position by calculating from their own figures and those of other observers the basal metabolic rate during pregnancy on this assumption. The weight of the fetus was calculated from the fetal growth curves, and Lissauer's formula for infants used to calculate its surface area. The weight of the woman was estimated from her pregnant weight minus the weight of the fetus, and the surface area determined by the Du Bois charts. Thus the basal metabolic rates of the woman as a pregnant organism, and the fetus were calculated separately. The sum of the two agreed remarkably well with the figures actually found.

Pregnancy would thus appear to result in no alteration in the energy exchange beyond that produced by the growth within the maternal organism of a new mass of active protoplasmic tissue of a higher basal metabolic rate, and comparable to that in infants.

The series of experiments thus outlined would offer no support to the idea that a special hyperactivity of the maternal thyroid occurs during pregnancy. It may be noted, however, that the calculations of Zuntz and of Carpenter and Murlin showed an increased metabolic rate in the mother as distinct from the fetus of about 4 per cent.

MINERAL EXCHANGE. In a recent review in this series Shohl (11) has given a résumé of the mineral metabolism of the fetus and of pregnancy. Shohl has calculated the total absorption of the acid and basic ions in the form of 0.1 N solutions with the result that 11,970 cc. 0.1 N acid and 18,680 cc. 0.1 N base are found to be required by the

full-grown fetus. This includes P. S and Cl as acid, and Na, K, Ca, Mg as basic radicles. The excess of basic radicles amount to 84 cc. 0.1 N base a day reckoned over the last 8 weeks of fetal growth an amount sufficient to account for the disturbance of acid-base equilibrium known to exist in pregnancy (vide p. 295).

The total balance of P, S, Ca and Mg during pregnancy has been observed by Hoffstrom (30) and Landsberg (31). Large positive gains in these elements have been observed to be made by the mother independently of the fetus, again presumably to act as reserve material during the period of lactation. The position with regard to Ca retention by the mother is doubtful. Some studies on the effect of iron-rich and iron-poor diets have been made by Fetzer (57).

N PARTITION IN THE URINE. Owing to the many erroneous views which have been held from time to time on the function of the liver and kidneys, and to an incomplete knowledge of the distribution of the total N of a urine among its various components, claims have been made on the value of this or that fraction as diagnostic of a disturbance peculiar to pregnancy. Thus the amount of ammonia, urea, creatine or undetermined N has in turn been the subject of a hasty conclusion. The urine in normal pregnancy is identical with that of the non-gravid individual under similar conditions, with the exception of the occurrence in it of creatine in the later stages of gestation. A pregnant woman excretes less N on the same diet as her non-pregnant control, has less urea and hence a larger percentage of ammonia and creatinine. This is, however, merely because of the N gains which are made in pregnancy, and the remainder of the changes are in accordance with the laws enunciated by Folin (58). The details and summaries of some of the discussions may be gained from the papers of Underhill and Rand (59), Murlin and Bailey (60), and Losée and Van Slyke (61). The occurrence of creatine in the urine of pregnancy has been observed in dogs by Murlin (62) and in women by Shaffer (63). This is but one aspect of the metabolism of creatine on which recently a review has appeared in this series (64). One may note, however, that creatine occurs in the urine in conditions of either a positive or negative N balance.

THE EQUILIBRIUM IN THE BLOOD. *Hydration.* It has been recognised for a long time that the blood in pregnancy is more dilute than in the non-pregnant condition. Nasse (65) in 1876 came to that conclusion from a study of the specific gravity. Lloyd-Jones (66) made similar observations and conclusions. Zangemeister (67) made many varied

determinations and found a lowered specific gravity, diminished total solids, a lowered depression of the freezing point, and a lessened percentage of serum proteins, all indications of an increased hydration during pregnancy. Landsberg (68), Dienst (69) and Eckelt (70), and Plass and Bogert (71) also studied the percentage of plasma or serum proteins, and De Wesselow (72) examined the percentage of total solids during the period of gestation. Stander and Tyler (73) have followed the moisture and ash content of plasma during pregnancy. The results of these researches all point in the same direction. Indeed, the dilution of plasma would appear to be quite an early change due to pregnancy, for Plass and Bogert observed a slight lowering of the average percentage of plasma protein in the first two months. By the third month the increase in the hydration is marked. These same observers, from a series of average values, state that the hydration of the plasma increases until the sixth month, when it gradually decreases until labor supervenes, though it never reaches the average non-pregnant level.

Blood volume. The diluted state of the blood would appear to be caused by a true increase in blood volume, at any rate, in the later stages of pregnancy. Miller, Keith and Rowntree (74) by an injection of vital red observed an increase in both relative and absolute volumes of the maternal blood independent of the fetus or its appendages, for they were unable to find the dye in the placenta, amniotic fluid or infant's urine, when injected during labor. Koch and Jakobvitz (75), using a solution of congo red as the injection fluid, were unable to note any increase in blood volume. Gueissaz and Wanner (76), using the method of de Crenis and Löwy (injection of glucose and determination of refractive index of serum), however, noted a 15 per cent increase in pregnancy, and Kaboth (77) calculated an increase of 300 to 450 grams of blood in pregnant women approaching term.

The state of the capillary system has been examined (using the method of Lombard) by Nevermann (78), Hinselmann (79), and by Baer and Reis (80). The capillaries in pregnancy, however, would appear to be similar in character to those of the normal non-gravid individual.

Corpuscular elements. According to some unpublished work of Harris (quoted by Williams (24)) the increased blood volume is associated with a decreased red cell count and hemoglobin content. Gram (81) also claims the percentage of hemoglobin to be less in pregnancy falling to a minimum at the fifth month and then rising to almost non-pregnant level at term. He regards the "anemia of pregnancy" as a continuation of this normal change. A leucocytosis is observed just before labor.

Fibrinogen. The fibrinogen content of the blood rises in pregnancy. Gram (82) has recently published a monograph on this subject and its relation to pregnancy. De Wesselow (72) found an average value of 76 mgm. fibrinogen N per 100 cc. blood, whereas the normal non-pregnant value averages as 40 mgm. There is an increase in the later months compared with the early months.

Non-protein N and urea. Since the advent of the new methods of blood analysis inaugurated by Folin there have been many investigations on the blood in pregnancy dealing with these figures, the great majority of which have appeared since 1920. The reasons underlying these researches have been twofold.

1. To establish a series of figures for normal pregnancy, comparing the pregnant and non-pregnant conditions.

2. To compare the figures of the different months of pregnancy, in order to detect any characteristic variation.

The first object may be said to be definitely settled for the values of the non-protein N. The values in pregnancy are similar to those found in the non-pregnant. Perhaps they may, on the whole, be slightly lower. Thus Folin (83), Hellmuth (84), Williams (85), King and Denis (86), Bunker and Mundell (87), Harding, Allin and Van Wyck (88), rarely find figures of over 30 mgm. per 100 cc. blood. Caldwell and Lyle (89), who used trichloracetic acid as the protein precipitant, found many cases with values higher than this, and their average maximum figure would appear to be around 35 mgm. Further figures may be found in other papers, and a tabulation of many of these is given by Harding, Allin and Van Wyck.

The figures for urea are not so well investigated. They are sufficient, however, to make it clear that, compared with the non-pregnant condition, the ratio urea N: N. P. N. is lower in pregnancy. The only question which arises is the extent of the diminution. In the non-pregnant normal individual this ratio is accepted for whole blood at 50 per cent. In pregnancy the average ratio has been given as 44 (Killian and Sherwin (90)), 39 (Caldwell and Lyle), 35 (Williams), 30 (De Wesselow (91)), 25 (Folin), and various suggestions have been made on the significance of the lowered values. Folin first suggested an increased permeability of the kidney, and one notes with interest that application by Spalding, Shevky and Addis (92) of the Addis test for the functional activity of the kidney, which depends upon the use of urea, gives results indicating an increased sensitivity in the pregnant condition. The less sensitive test of MacLean and De Wesselow gives urea concentrations of

normal value. The dilution of the blood which occurs during pregnancy has been supposed to be the cause of the low urea levels, but the supporters of this explanation neglect the fact that the blood is not a closed system, being in equilibrium with the tissues, and that the kidneys are actively passing urea into the urine. Indeed, De Wesselow himself notes that in the hydremia of pure parenchymatous nephritis, the blood urea levels, though low, do not fall as low as in pregnancy. Diminished production of urea has also been brought forward by Caldwell and Lyle and De Wesselow as an explanation, and in view of what we have noted on the N balance in pregnancy this factor certainly cannot be neglected. The absorption of 2 grams or more N a day must lower the excretory N taken as an average value. Dietary factors, however, will profoundly influence the figures, and until these are known in pregnancy with greater exactitude, the part played by diminished production of urea in this phenomenon must remain uncertain. Caldwell and Lyle have also considered an increase in the undetermined N as a partial cause for the lowered urea N percentage.

Very little attention has been paid until just recently to the effect of the period of gestation on the figures. Caldwell and Lyle noted no effect upon the N. P. N. or urea values except to observe an increase in the N. P. N. in the month preceding labor. This was not observed in Williams' series, but Harding, Allin and Van Wyck noted a gradual increase in the N. P. N. values, culminating at term. The figures of Bunker and Mundell show a slight drop in the middle of the gestation. The urea during gestation is constant according to Caldwell and Lyle, and Bunker and Mundell, but shows lower values in the middle of gestation according to Williams. The differences, however, are slight, and it is probable that the number of cases studied is not sufficiently large to exclude variations due to dietetic differences. Harding, Allin and Van Wyck have also suggested the possibility of seasonal variations.

Uric acid. Many of the papers to which reference has been made in the last section also contain figures on the uric acid content of blood in pregnancy (84), (86), (87), (88), (89), (90). The values are very similar to those found in the non-pregnant condition, ranging, from 1.7 to 3.5 mgm. per 100 cc. blood. Although the lower limit is hardly ever reached there are many figures higher than the maximum just given which must be accepted as normal. A seasonal variation probably exists, and here there would appear to be little doubt that the values increase as gestation progresses, culminating in labor. The figures obtained by different observers point in the same direction, though this

is not equally evident in all sets of observations. The significance of this increase is not clear, but it need not necessarily be interpreted as a sign of renal dysfunction. Thus Harding, Allin, Eagles and Van Wyck (93) have just shown that marked increases in blood uric acid can be produced by the simple expedient of giving a diet high enough in fat to produce ketosis.

Amino acid. The value of this fraction of the N of blood seems to be within normal limits, the values found lying between 5 and 8 mgm. (Losee and Van Slyke (61), and Morse (94)) amino acid N. Neither is there to be found any variation (other than that of the nongravid individual) in the polypeptide N of the blood during pregnancy.

Creatinine. The values for this substance lie within the usual normal range (84), (86), (87), (90). Bunker and Mundell found no variation with the period of gestation.

Creatine. The values for this substance are most likely incorrect, as the analytical method was shown by Hunter and Campbell (95) to be subject to great error, especially when the determinations are made on whole blood. Such values as have been obtained are at term (84), (95).

Inorganic. With the exception of calcium only a few determinations on the content of the inorganic constituents of blood are available. The prominence given to calcium determinations has arisen from the problem of bone formation in the fetus, and the occasional occurrence of osteomalacia in, or arising, from pregnancy, together with theories which have been held on the origin of the convulsions of eclampsia. The results of the investigations, however, go to show that the level of the blood calcium is little, if any, disturbed. Such variations as have been found are almost within the accepted normal limits and are only detectable from a study of averages. They are not comparable with the changes observed in rickets or in severe nephritis. Nor is there a unanimity of opinion on their character. Bell and Hicks (96) claimed an increased amount previous to labor and correlated this with the mechanism of labor itself. Only Lamars (97), has found a similar result. Jansen (98) and Denis and King (99) found no significant changes in the Ca content of the serum throughout pregnancy, nor did Underhill and Dimick (100) claim any in whole blood. Kehrer (101), in whole blood, De Wesselow (72), Bogert and Plass (102), and Krebs and Briggs (103) in serum, observed a slight average decrease in later pregnancy, as also did Widdows (104) who followed a few individual cases. De Wesselow also observed a slight decrease in the P content of

the serum. The average results of the concentration of the inorganic constituents both of serum and whole blood in pregnancy are collected in table I.

The figures as presented in this way would appear to show that Na decreases slightly in concentration in the serum and increases in the corpuscles, and similarly with Ca and Cl, whereas K, Mg and P remain unaffected. Such changes, however, are slight, are not outside the normal range of variation, and any conclusions can only be drawn in the most tentative manner. An increase in chlorides of whole blood in pregnancy is claimed by Bunker and Mundell (87), and some unpublished results from this laboratory would indicate that these figures reach a maximum at the sixth or seventh month, falling just before labor, and making the curve of chloride concentration during preg-

TABLE I

Inorganic constituents of blood in pregnancy

	Na	K	Ca	Mg	Cl	P	S	
	Mgm. per 100 cc. serum or 100 grams whole blood							
Non-pregnant*.....	339	20.5	10.0	2.4	360	3	0.5	Serum
Pregnancy†.....	325	21.5	9.5	2.4	338	3	0.5	Serum
Non-pregnant‡.....	182	19.2	6.9	2.7	286	11		Whole blood
Pregnancy‡.....	205	19.9	7.6	2.4	292	11		Whole blood

* Figures from Denis and Hobson.

† Average figures compiled from data of Krebs and Briggs, and Denis and King.

‡ Figures from Underhill and Dimick.

nancy opposite in character to that of the plasma proteins as shown by Plass and Bogert (71).

Acid-base. Although there may be uncertainty about the changes in blood just mentioned, yet it is impossible to doubt the fact that the acid-base equilibrium is disturbed in pregnancy with the production of a compensated acidosis. It was first noticed by Hasselbach (48), and in the same year by Liemdörfer, Novak and Porges (105), that the aveolar CO_2 tension was lower in pregnancy. The classical paper, however, on this subject is by Hasselbach and Gammeltoft (106) in 1915. They found the alveolar CO_2 tension in the gravid condition to be 30 to 35 mm. Hg. Moreover, they were able to observe the course of the tension in one individual from a pre-pregnant period to the puerperium. Pregnancy showed a lowering of CO_2 pressure even as early as the second

month. The pH of the serum also showed an increase in a series of cases taken toward the end of pregnancy, and compared with the same series after term. Losee and Van Slyke (61) showed the CO_2-combining power of the plasma to be definitely lowered during pregnancy. Cook and Osman (107) have found this to be true as early as the third month. Rowe (108), in a long series of cases, found the alveolar CO_2 tension to average 32.6 mm. No adequate explanation of this change has been offered. Shohl's explanation (p. 289) could not apply to the early months of pregnancy for, as shown in figure 2, the fetus takes a negligible amount of salts from the mother until the sixth month.[1] Marrack and Boone (109) adduce evidence to show that the lowering of the CO_2 tension is a primary effect and not due to the production of an unknown acid. In a few of their cases they showed an actual alkali deficit.

Carbohydrate. The amount of glucose in the blood in pregnancy does not differ from the non-pregnant condition, showing the usual range of 0.08 to 0.12 per cent. A recent article by Rowley (110) contains a resumé of previous observations. Nor does the progress of pregnancy cause any characteristic deviations within the normal limits (87). The giving of 100 grams of glucose causes a characteristic rise and fall in the blood sugar, similar to that found in normal non-pregnant individuals, though Bauer (111) found the point of maximum concentration of the blood sugar curve to be 0.2 per cent in a series of 200 pregnant women. Nevertheless, although the blood sugar curve behaves in a normal manner, yet the majority of pregnant women to whom this test is applied show a glycosuria. So characteristic did Frank and Nothmann (112) consider this that they proposed it as a test for pregnancy. Undoubtedly the majority of gravidae react in this manner, especially in the first trimester (113), (114) although the occasional exception is found. Later in pregnancy the value of the test becomes much more doubtful. Similarly Kamnitzer and Joseph (115) have also inferred the increased permeability of the kidney for sugar in pregnancy through their phlorhizin test which depends upon the production of a glycosuria with amounts of phlorhizin much smaller than those requisite to produce it in the non-pregnant condition. A large number of papers on this subject from the clinical aspect have recently appeared confirming the general nature of Kamnitzer and Joseph's findings, but again there are sufficient exceptions to make the test valueless as a critical one (116).

[1] In a private communication Dr. F. Cook informs me that he has observed the lowered CO_2 combining power of the plasma as early as three days after the first missed menses.

The usual relations of carbohydrate and fat are also held to be disturbed during pregnancy. Thus Porges and Novak (117) found that a diet free from carbohydrates produced a ketonuria in a few hours. Gilliatt and Kennaway (118) observed higher ammonia production in nausea and vomiting of pregnancy than that usually found in starvation. Pritzi and Lichtmann (119) used the conditions of Porges and Novak and found positive early ketonuria in 47 out of 57 pregnant women examined. Strict quantitative observations are lacking, however, and Harding, Allin, Eagles and Van Wyck (120), in a note just published on this subject, draw their conclusions with extreme caution, on account of the many assumptions that have to be made.

Lipoids. The lipoids in general alter in concentration in the blood during pregnancy. Fat, lecithin and cholesterol increase. Mainly owing to the fact that cholesterol represents a well-defined compound and is a more easy determination than either fat or lecithin, attention has been almost exclusively directed to this substance. Cholesterol increases in the plasma throughout pregnancy (121). Huffmann (122) showed that a maximum was reached in the last months, followed by a return to normal figures 10 days post-partum. Changes in the corpus luteum, or a deficiency of liver function (123) have been suggested as the origin of this change. Slemons and Stander (124) viewed it together with the increase in fat and lecithin as a preparation for lactation. Some color is lent to this view by the observations of Hermann and Neumann (125) that non-nursing parturients do not show so rapid a fall in the lipoid content or blood as lactating women, though this may merely be the effect of the closing of one of the channels of excretion. Lindemann (126) also showed the increase in fat and lecithin. Two recent papers by Pribane and Pribram have shown that increase in cholesterol in the blood is accompanied by a decreased excretion in the bile and that the excess is disposed of after labor, through the bile (127) and also through the mammary gland (128).

PLACENTAL INTERCHANGE. Because of its relation to the general philosophy of "vitalism" as well as to the more immediate question of the nutrition of the fetus, the study of the passage of substances through the placenta has acquired a special interest. Excellent reviews have been published from time to time, notably those of Nicloux, (129), Murlin (130) and Slemons (131). Recent summaries are also given by Underhill and Amatruda (132) and Williams (24). Nicloux divided substances into two classes, those soluble in water and permeating the placenta, and those insoluble in water to which the placenta presented

a non-permeable barrier. This was based on the behavior of simple drugs as iodides and salicylates, of alkaloids such as strychnine, pilocarpine, of the anesthetics, and of gases such as CO_2 and O_2, all of which were found freely on both sides of the placenta, whereas fats were found to be without influence on the fetal blood (133). The division, however, has been shown by later workers to be by no means so simple and clear-cut; indeed, it is very possible that a repetition of some of the older experiments on a quantitative basis would reveal marked differences between some of the diffusible substances. Thus Cunningham (134) has shown that two substances such as sodium ferrocyanide and ferric ammonium citrate, both of which are freely permeable to inert membranes, behave differently toward the placenta when administered to the pregnant cat. Both "penetrated the syncytial layer of the chorionic ectoderm in about two hours and finally the ferrocyanide reached the fetal circulation in four to five hours, while the citrate had not done so at the end of ten." Trypan blue was found to be retained by the same layer of cells by Wislocki (135), and neoarsphenamine was found to be stored by the placental tissue after maternal injection but not by fetal tissue, although a small constant amount of As was observed (132). Does the placenta behave in a selective way toward those normal constituents of maternal blood upon which the fetus must ultimately depend for its growth, and is it freely permeable to the end-products of metabolism? This is a view which would seem to be gaining ground. Fats undoubtedly exist at a higher concentration in maternal than fetal blood. This is shown by Slemons and Stander (124) in figures for total fat, lecithin and cholesterol, both free and as ester. The position of lecithin is confirmed by Plass and Tompkins (136) who found a similar distribution for lipoid P. It would thus appear that the fetus manufactures its own fat, presumably from carbohydrate, as fat stained with Soudan III is not observed to cross the placental barrier. Bogert and Plass (137) have noted a slightly higher concentration of Ca on the fetal side of the placenta, and Plass and Tompkins similarly for inorganic P. Edelstein and Ylppo (138) find Na and K higher in fetal blood than in maternal blood, in all cases examined but three. Magnesium, however, has the same concentration. Morriss (139) found constantly a greater amount of sugar on the maternal side, and in this he is confirmed by Rowley (110) and by Howe and Givens (140). The differences, however, are very slight. Amino acids also exist unequally distributed, both Morse (141) and Bock (142) finding a higher concentration in the fetal blood.

On the other hand Kingsbury and Sedgewick (143) and Slemons and Bogert (144) find uric acid evenly distributed between the two bloods. Plass (145) and Hunter and Campbell (95) find creatine and creatinine in plasma, and Slemons and Morriss (146) find urea to be of the same concentration on either side of the barrier. The substances circulating in blood thus divide themselves very conveniently into those useful in nutrition, and those of excrementory character, with regard to their diffusibility through the placental membranes. Howe and Givens have criticised this view and in a series of analyses of the two bloods have noted differences in concentration of urea quite equal to those found in substances which belong to the class over which the placenta is supposed to exert a selective action.

PUERPERIUM. Physiologically the puerperium is marked by two changes, the involution of the uterus, and the onset of lactation. With the second this review has little to do. The first, however, is regarded as the cause of the losses of N which characterise this period. This explanation was given by Slemons (147). The phenomenon was observed by many of the early workers in the field of N metabolism in pregnancy and many of the papers cited under that heading in this review contain figures and references. Slemons' conclusions were reinforced by his experiments comparing the N losses in simple Caesarean section with those of complete removal of the uterus. The N loss was found to be greater in Caesarean section by about 2 and 3 grams a day. In these and in other experiments of a like nature, however, the results are confused by lack of adequate dietary control. The involution of the uterus too has been assigned as the cause of post-partum creatinuria, first observed in women by Shaffer (63), and in dogs by Murlin (62). Mellanby (148), however, believes the creatine to be produced paripassu with the progress of lactation. Morse (149) agrees with Mellanby that the involution of the uterus does not represent the origin of the creatinuria, but neither does he believe it to be connected with lactation.

Ten days from delivery the changes which have been described as characteristic of the pregnant period have disappeared. The N balance may become negative due to the demands of lactation; the basal metabolism becomes normal, or perhaps subnormal, due to lessened activity. Blood volume has been reduced, and the plasma proteins restored to their original concentration. The uric acid in the blood sinks to its usual level, and the CO_2 tension of alveolar air rises to its pregravid height. Cholesterol becomes normal. These changes thus

appear to be caused by the growth of a mass of embryonic tissue in an adult organism. In how far is this a general reaction illustrated by pregnancy?

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BLOOD PRESSURES IN MAN UNDER NORMAL AND PATHOLOGICAL CONDITIONS

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Long after accurate measurements of blood-pressure had been practised on experimental animals, the study of blood-pressure in man remained virtually a sealed book. Various early methods were tried without reliable results; it was not until Riva Rocci (112) and somewhat later L. Hill and Barnard (63) introduced the armlet method that systolic pressure estimation became really practicable. Even after this method had superseded the earlier attempts of Mosso, Gaertner, Von Basch and others, many results were rendered more or less inaccurate by imperfection in technique, too narrow armlets, etc., while the reliance on systolic pressure alone gave very inadequate and often misleading information as regards the state of the circulation. The later development of diastolic pressure estimation, especially by the auscultatory method, marked a great advance in the usefulness of the study of blood pressure. The adoption of the standard breadth of armlet or cuff as a result of Von Recklinghausen's (109) work was an important step.

As regards oscillatory methods the technique and the principles involved have lost much of their interest and relevancy, since their practical application has receded in importance in view of the development and general adoption of the auscultatory method. The superiority of the latter has become widely recognised, on the grounds of simplicity, quickness and accuracy, as compared with the more cumbrous apparatus and the more difficult and variable interpretation of the oscillatory records, different readings of pressure often being made from the same records by different observers of considerable experience or even by the same observers at different times—difficulties examined by Melvin and Murray (97) and others. A good many workers using oscillatory methods have found the Pachon oscillometer with its visual indications preferable to the Erlanger apparatus with its graphic records. The more recent Pachon apparatus has a Gallavardin armlet

with two independent pressure bags applied to the upper arm instead of the wrist; this is the best form of oscillatory apparatus at present available.

Experience has emphasised the importance of combining the tactile systolic index with the auscultatory systolic as a *routine procedure*, recommended by MacWilliam and Melvin (90) in 1914; the latter index should always show a higher value when the estimation is correctly made. This of course necessitates the use of one of the various forms of apparatus that provide for the retention of the auditory receiver in position, leaving the hands free, e.g., the Baumanometer, the Tycos, the Laubrey sphygmophone, the Oliver tambour, etc. The checking of the auscultatory systolic by the tactile systolic is essential for more than one purpose—as a guarantee of the proper functioning of the auditory apparatus, and in guarding against error which may sometimes arise after prolonged or repeated armlet compression. Repetition may be needed to correct the disturbances in pressure due to excitement, etc., at the first compression; as is well known, subsequent readings are frequently decidedly lower—until a constant level is reached, the residual pressure. Some persons under pathological conditions are specially liable to show a decided rise of pressure from repeated or continued compression especially when the arm becomes congested distally to the compressing armlet. Effects (reflex, etc.) from repeated compression by the armlet come into question, described by Gallavardin with Haour (53) and Tixier (54) as involving different types of pressure changes, rises, falls, etc.

The present writer has found important disturbances of the auditory indications in a certain number of subjects, as a result of compression which involves marked turgescence of the hand and forearm—a cutting down of the systolic index with a rise of the diastolic. Sometimes at a later stage of prolonged compression there is enfeeblement or disappearance (at variable points) of all the sounds below the upper region of sound, in the neighbourhood of the systolic level. Such disturbances may occur while the actual blood pressure is not changed —as shown by the tactile systolic index remaining unaltered; thus incorrect measurements including an unduly restricted pulse pressure may be obtained in these cases by the use of the auscultatory method alone—unchecked by the tactile method. Some subjects are exceptionally susceptible to the development of such disturbances; the significance of these differences in behaviour has not been determined.

Another purpose for which repeated compression has been used is to

reduce strong tonic contraction which may sometimes be present in thickened arteries, giving a certain resistance to obliteration and leading to an over-estimation (to some extent) of the actual intra-arterial pressure. But a better method is to close the brachial artery for three minutes by digital pressure upon it, as recommended by MacWilliam and Melvin, instead of constricting the whole limb by the armlet and leading to venous turgescence and the associated tendency to error described above—as especially applicable to a certain (limited) number of subjects. Experiments (unpublished) in this Laboratory show that in some instances at least the trouble is caused mainly by congestion of the limb, not simply by pressure on the brachial artery. Digital compression while removing the abnormal resistance to obliteration of the pulse does not induce the auscultatory error referred to above.

When the auscultatory method is rendered difficult by noisy surroundings or by impaired hearing in the observer, the vibratory method of Ehret (38) a modification of the tactile method, can be usefully combined—a finger being applied to the artery on the distal side of the auditory tambour, to detect the vibration associated with the sound at the diastolic level. This method is strongly recommended by Gallavardin (51). It is much simpler than checking by oscillatory methods. But difficulties are present in fat subjects with deep brachials, small calibre, and cases (especially aortic regurgitation) where the change in the vibration constituting the diastolic index is less definite than usual.

As regards the mechanism of the sounds, the subject of various conflicting views (Gittings (55), Erlanger (39), MacWilliam and Melvin, L. Hill and others) there have been interesting investigations by Gallavardin and Barbier (52), (8) who describe two zones in the curve of sound; 1, in the upper half of the curve with maximum near the systolic index and murmurs caused by whorls in the blood current as it passes through the compressed area of the artery and gets into a region of lower pressure distally to the armlet; 2, in the lower half of the curve with maximum near the diastolic index, the sounds here originating in the vessel wall and related to sympathetic nerve influence on the arterial wall. Attempts to interpret the meanings of the notable variations in the character of the sounds in different subjects, the duration of the phases and the changes induced by armlet compression have been made by Gallavardin with Haour, Barbier (52) and Tixier, and by Tixier (126), B. Smith (116) Sorapure (117) and others, following

observations by Ettinger, Goodman and Howell (57), (130) Warfield and others. The characters of the sounds in different conditions are so varied and striking that useful information as to circulatory states may very possibly be derived from them when they are better understood.

It need hardly be emphasised that the Hg manometer is the reliable means of measuring pressures; it is only when frequently checked against this instrument that other forms (aneroids, etc.) can be taken as giving valid evidence. In regard to diastolic pressure it may be noted that the reading taken when the armlet pressure is being raised is often appreciably lower (5 mm., etc.) than when taken during deflation; in some subjects the difference shows more than in others. With reference to the systolic index the difference in the readings by the auscultatory method and those by the tactile method have been estimated at 5–14 mm. The experience of the present writer agrees with the lower values, usually only a few millimeters.

Blood pressures in young adults. In 1914 Melvin and Murray (97) established by accurate methods normal values of both systolic and diastolic pressures in healthy young male adults (sitting posture), 59 medical students, average age 20–9. As regards systolic pressure only three were up to 130 mm. (viz., 130, 134 and 135) while five were slightly below 100 mm., the average came out at 112 mm. Of the diastolic pressures 28 were at 60 to 70 mm., 19 at 70 to 80 mm. and 12 at 50 to 60 mm. The pulse pressures gave an average value of 46 mm. Subsequent observations on very large numbers of subjects by various observers in different parts of the world have given S. and D. values higher as a rule and sometimes with wider ranges of variation. Bearing on this difference the observations of Alvarez (4) and of Burlage (23) (to be stated presently) as to a lowering of pressure in the early years of adult life are suggestive, as they include the ages dealt with by Melvin and Murray. Sorapure (117) examining 769 British soldiers also found a systolic maximum at 19 to 22 years followed by a slight fall, as Stocks and Karn (121) did after a systolic maximum at 19 to 20. It may be remarked that while large numbers are of course necessary for statistical purpose, classification, etc., reliance on pressure measurement on a single occasion is apt to introduce sources of error, in view of the universally recognised tendency of single examinations to give results disturbed by temporary causes, nervous excitement, etc. More precise results, as regards the real pressure levels in individuals are obtainable by a more intensive study of smaller numbers, by repeated examinations under carefully ascertained and controlled conditions.

Among more recent investigations Alvarez (4) and his associates made observations on systolic pressure (tactile method) on a very large number of University students (6000 men and 8934 women). In women the average was 11 mm. lower than in men. In men (reclining after tepid shower bath) the pressures grouped mainly about 127 mm. at age of 16 and 118 mm. at 30; in women (standing) 118 mm. at 16, 111 mm. at 24, 117 at 40. There is thus a noteworthy lowering of systolic pressure in the early years of adult life, the average dropping from age 17 to 21 in men and remaining at about the same level till after 50; in women falling from 17 to 25, rising after 25 and especially after the age of 40. After 45 the average pressure is higher in women than in men. A fall of pressure was also noticed by Burlage who made observations on 1700 girls by the auscultatory method; he found a systolic pressure of 104 mm. at 9 years, 124 at 14 and 15, falling to 114 mm. at 18, then constant to 26. Alvarez notes that relatively high pressure is common in young men—45 per cent over 130 mm., 22 per cent over 140 mm.; in young women, 12 per cent over 130 mm. and 2 per cent over 140 mm. Evidence of the occurrence of comparatively high pressures in some young men is also to be found in the results of Barach and Marks (7), Lee (81) and others. Alvarez pronounces his extended investigation as not entirely satisfactory in establishing normal systolic pressure standards for young men on account of the lack of homogeneity, without arriving at any definite explanation of the results, the possible causes of which he discusses—low pressures in 1918, raised in 1919, gradual return in 1920–21. Further there is the disturbing observation that the averages even in 1918 were considerably above those of high school boys of the same age in the same year.

Conception and Bulatao (29), examining 717 subjects (average age 28) in the Phillipines, found in males S.115, D.79; in females S.116, D.83. Pulse rate a little over 72. In Denmark, Faber (43) in 1000 healthy soldiers (ages 20 to 25) by the Riva Rocci method (recumbent) found S. pressures of 110 to 130 mm. in 80 per cent, and higher or lower, 84 to 156 mm., in 20 per cent. Emphasis is laid on these great variations of systolic pressure in healthy men. Men of greater weight showed higher pressures than others of the same height; with equal weights blood pressure is lower, though the differences were slight, in men of greater height. What are termed the "overfat" averaged 123 mm. as compared with 117 mm. for the "underfat." Diastolic pressures are not recorded. The systolic pressures found by Faber agree with those obtained by Tavaststjerna (124), whose average was 117 mm.

Addis (1) examined nearly 400 subjects in two categories, the pressures being taken in the recumbent posture—1, under basal conditions; systolic average 99, diastolic 71. 2, Under other conditions, food taken, walking, etc., systolic average 127, diastolic 78.

The relation of blood pressures to age. There is general agreement as to the presence of lower systolic and diastolic pressures in childhood, the differences from the adult being more marked in the systolic levels with a consequent diminution of the pulse pressures.

The work of Judson and Nicholson (71), Melvin and Murray, and Faber and James (44) may be referred to, also the more recent observations of Stocks and Karn (120). In connection with the smaller pulse pressures the quicker pulse rate of children has to be taken into account, tending to make the product of P.R. × P.P. approximate to what holds good in the adult.

The available evidence shows that from the very early phase of life there is a progressive steady rise of pressure, apparently a function of increasing age, up to the onset of puberty, then an acceleration of the rise up to the ages of 17 to 20. It is to be noted that there are decided differences between the results of Judson and Nicholson and of Faber and James on American boys, and those of Stocks and Karn on British boys, as regards the actual values of the pressures recorded and the extent of the rise between the ages of 5 and 14 years. At the former age the American observers found systolic averages of 92 mm. and 93 mm. respectively; at the latter age 106 mm. and 110 mm. On the other hand Stocks and Karn report a lower average, 85 mm., at age 5 and a higher level, 115 mm. at age 14—a rise of 30 mm. which is nearly twice that found by the other observers. The accelerated rise during puberty and adolescence between the ages of 13 and 17 has been found by Stocks and Karn to amount to 16 mm.

Woley (134) dealing with systolic pressures in 1000 apparently healthy subjects, found an average level in males of all ages of 127.5 mm., in females of 120 mm., and a rise from 122 mm. in the age group 15 to 30 years to 132 mm. in the 50 to 60 age group. He distinguished a high pressure group with an average pressure of 141 mm. at the ages 15 to 30 to 149 mm. at 50 to 60, and a low pressure group rising from an average of 103 mm. in the 15 to 30 group to 115 mm. in the 50 to 60 category. At the intermediate ages averages of intermediate value were obtained, a gradual rise occurring with increasing age and a corresponding rise in high and low averages. He regarded a pressure of 144 mm. in the 50 to 60 age group as being definitely acceptable for

insurance. Women at all ages were 8 mm. below the male average, with the same ratio of increase at similar ages.

In Symonds' (122) report of 150,419 men successive age groups are presented from a 15 to 19 year group up to one of 60 years and over; also build groups (Medico-Actuarial Investigation I, 1912, 120) based on the average weight for each inch of height in men at the age of 37. Systolic pressure alone is studied. Age, weight and pressure are shown to increase together. Differences appear of 11 to 12 mm. between the youngest and the oldest in each build group, and of 10 mm. between the very light and the very heavy groups; even at the ages of 60 and over the difference was much the same. In the whole series the pressure averages range from 121.2 mm. in the youngest (15 to 19) group to 135.2 mm. in the oldest (60 and over) group. Mackenzie (87) reporting on 18,637 men, gives a range from 119 mm. to 137 mm. in similar age groups. Rogers and Hunter's (113) 62,000 rise from 120 mm. at 15 to 19 to 134 mm. at 55 to 59; the results of Fisher (46) and Goepp (56) are very similar though the age grouping differs slightly. As regards the pressures in women, Symonds' 12,000 with age grouping similar to the men show values ranging from 119.2 to 135.5—a much closer approximation to male pressures than has been found by most observers who have commonly reported pressures in women, at least in the first half of life, as 8 to 10 mm. lower than in men.

In a recent valuable study Stocks and Karn (120) present continuous evidence of the pressure behaviour through the ages of puberty and adolescence, from the ages of 5 to 40 years. They submit curves and tables for the correction of pressure readings for age, weight (affecting systolic pressure) height (affecting diastolic pressure) and pulse rate. They find that there is a positive correlation of systolic pressure with muscular strength apart from physical development and age. By their method of correlation with pulse rate they believe that an approximate correction of the well-known disturbing effects of psychological factors, such as nervousness, can be made. With regard to this conclusion of Stocks and Karn it is to be remarked that excitement, emotion, etc., influence blood pressure by acting on the vasomotor centre as well as on the heart, and that the relation between the two actions is by no means constant; hence the pulse rate cannot be relied on to give accurate indications of the degree of pressure alteration developed, though a correction for pulse rate no doubt diminishes the amount of the error.* The range of apparent variability of the blood pressure in healthy persons is substantially diminished by such cor-

rections as the preceding, though not removed, since other and more obscure factors remain. Stocks and Karn's figures show a remarkably small increase of pressure with advancing age—from an average of 131 mm. at 20 years to 134 mm. in the group of 40 years and over (average age, 49).

It is warrantable to conclude from the concurrent evidence of the extended statistical evidence now available that the idea of an extensive progressive rise of systolic pressure in healthy persons as life goes on is an erroneous one. It is clear that the rising pressure of childhood undergoes accleration about puberty and attains what is approximately the adult level somewhere in the 17 to 20 period. There is some evidence of a slight subsequent lowering—in the early years of adult life. Apart from this, the pressure remains almost steady till the age of about 40, after which a more definite rise progresses. But the rise, though quite a definite one, is more limited in amount than is commonly assumed; the total rise shown in the statistics, due to the combined influences of age and increasing weight is on an average under 15 mm. The pulse pressure follows a course pretty similar to that of systolic pressure under the influence of age. The available evidence also bears weighty testimony to the relative constancy of systolic pressures at different ages, when large numbers are dealt with and the necessary allowances and corrections are made. It is clear, in view of the foregoing averages, that the occurrence of exceptionally high pressures in healthy persons must be relatively rare; otherwise the averages would be much higher. The very moderate level of the averages for middle and advanced life is all the more noteworthy in view of the fact that the real ordinary pressures are likely to be over-estimated rather than under-estimated, under the influence of nervous excitement, etc. But while the averages for large numbers are relatively constant, the fact of notable variation in healthy individuals remains, the pressures in such persons being apparently set at levels different from the ordinary—from causes that cannot at present be adequately defined. The importance of hereditary influences has been emphasised by numerous observers, e.g., Oliver (104), Dana (34), Alvarez, Warfield (130) and others.

With regard to the very high systolic pressures, S.200 to 250, sometimes (though rarely) met with in apparently healthy vigorous men at such ages as 50, 55, etc., the mechanism of such pressures is well worthy of careful investigation—with respect to the peripheral resistance, capillary and venous pressures, blood volume, cardiac output,

etc.; such would probably yield valuable information as to circulatory conditions. Various observers have noted that in a considerable number of high pressure cases there are no definite symptoms and no evidence of disability; Kulbs (77) found so high a proportion as 20 per cent in this category in a series of 172 males and 116 females with pressures at or above 170 mm. As regards diastolic pressures the American life insurance data are open to criticism from the adoption of the end of the 4th phase as the diastolic index, the latter having been experimentally proved to coincide with the beginning of the 4th phase —in the dog by Warfield (129) and in the sheep by MacWilliam, Melvin and Murray (91). It is certain that serious error may occur in this way especially in young subjects where the duration of the 4th phase may in some cases be long. Thus Melvin and Murray, by careful examination in quiet surroundings using a sensitive Oliver auditory tambour, found in 14 young men out of a total of 59 a prolonged 4th phase, ranging between 24 and 55 mm. and averaging 38 mm. The lower limit of the sound was sometimes found to be as low as 10, 14, 20 or 22 mm. armlet pressure in healthy subjects with normal systolic pressures and complete absence of any collapsing character in the pulse, etc.; obviously these figures could not possibly represent the actual diastolic pressures.

Of course such very low readings of the lower limit of the sound are exceptional. Many observers have noted a 4th phase of shorter duration, e.g. Warfield up to 20 mm., Weysse and Lutz (131) not above 25 mm., Tixier usually 20 to 30 mm. at ages of 20 to 30 years and in some abnormal subjects 20 to 40 mm., etc. Others have reported figures 5 to 8 mm. (Goodman and Howell, Barach and Marks, Mackenzie, Smith and others). In middle-aged and elderly subjects the experience of the writer is that the sound rarely persists in any important degree (not more than a few millimeters) and consequently the lower limit of sound in these subjects approximately indicates the diastolic pressure—in contrast to the serious discrepancy which may occur in young persons.

In the insurance statistics referred to the relatively large numbers in the younger groups of subjects (19 to 25 and 25 to 30) would naturally tend to give scope for possible errors in this direction. But such errors would be in the direction of underestimating the actual diastolic pressure; on the other hand, the diastolic readings given in the insurance series referred to are by no means low.

There is sometimes a tendency to undervalue precision of blood-

pressure measurement and to regard differences of 10 to 20 mm. in blood-pressure readings as being of small moment in view of the larger variations that may occur from time to time with apparently little significance. But the importance of such differences varies greatly in relation to their position in the scale of pressures. When they start near the "normal" lower limits of systolic and diastolic pressures, differences of 10 to 20 mm. may mean much, e.g., between 100 and 80 or 90 systolic, or between 60 and 40 or 50 diastolic, such are of much significance as compared with similar amounts at higher levels.

Relations of systolic, diastolic and pulse pressures. The 3:2:1 ratio commonly cited as applicable to these pressures is subject to very considerable variations without coming into the category of the abnormal. The validity of the ratio is mostly evident with certain normal pressures, e.g., S. 120, D. 80, P. P. 40, the pulse pressure being one-half of the diastolic and one-third of the systolic. It does not hold good in such low pressures as may sometimes be found in healthy persons, e.g., S. 105, D. 60; where the P. P. is three-fourths, instead of one-half, of the diastolic and much nearer one-half than one-third of the systolic. Again with such a high diastolic as 120 mm. (muscular effort, etc.) a S. 180 and P. P. 60 are apt to be under what actually occur, the high diastolic tending to give a relatively higher S. and P. P. on account of the tense condition of the arterial walls; the discharge from an efficient L. V. causes a disproportionately large rise of systolic pressure—apart from the influence of an increased discharge per beat occurring in a distended or enlarged heart. Such effects of diminished distensibility of the arterial system at high diastolic pressures may also be paralleled by loss of elasticity and stiffening of the arterial walls from degenerative changes, apart from the presence of a high diastolic pressure. These factors being operative at different ages, it is evident that while the average (absolute) values of pulse pressure in large numbers of persons vary with age in the manner already stated, the actual amounts in individuals may be greatly affected, apart from the influence of age, in the ways just stated. Pulse pressures of deficient amounts associated with a high diastolic level are naturally of evil significance as indicating cardiac inefficiency, provided the low P. P. is not accounted for by acceleration of the pulse rate.

Blood pressure in muscular exercise. While a rise of blood pressure has long been known to be associated with muscular exertion, the great majority of the measurements have been made after the period of exertion has ended and have for the most part dealt only with sys-

tolic pressure. The methods adopted by Bowen (19) (systolic pressures only) and by Lowsley (86) (systolic and diastolic by the Erlanger sphygmomanometer) enabled the course of the pressure changes to be followed throughout the period of exercise (stationary bicycle). They found a rapid rise at first, reaching a maximum in a number of minutes, (Bowen, 5 to 10 minutes, Lowsley, 5 to 25 minutes) then slowly declining and after the end of the exercise sinking to normal or subnormal levels.

McCurdy (96) measured systolic pressure during brief maximal effort (heavy lifts involving thoracic fixation, etc.) lasting about 5 seconds, and found an average rise from 111 mm. before the effort to 180 mm. during and 110 after.

Measurements made "immediately" or at certain periods afterwards are not valid guides to the actual height of the pressure during the exercise, though such have been used by some observers in comparing the blood-pressure response to effort in different types of subjects. Thus some observers have taken estimations half a minute after the termination of exercise as a standard. Cotton, Lewis and Rapport (28) making repeated measurements found a fall to, or nearly to, normal in 10 seconds then a rise reaching a maximum in 20 to 60 seconds, there is then a gradual fall, reaching the resting level in from 1 to 4½ minutes after the end of the exercise (20 pound dumb-bells). Similarly Chailly-Bert and Langlois (27) recorded a fall in 5 seconds after the cessation of exercise to about normal, followed by a subsequent rise. Graupner (58) and Barringer (9) had previously described a secondary rise after the end of the period of exertion with a Zuntz ergometer and with dumb-bells respectively. These observations mainly deal with systolic pressures. The interpretation put upon their results by Cotton, Lewis and Rapport is that, assuming the veins to be depleted during the period of exertion, these veins fill up with blood when the muscular action ceases and so cut down the return of blood to the heart and the arterial pressure, until the veins have refilled and the inflow into the heart is restored, leading to the subsequent rise in presence of the continuance of the factors, other than the pumping action of the muscles, operative during the exercise.

This interpretation has been disputed by Bainbridge (6) who regards the veins as being full during the period of exertion, and the fall of arterial pressure to be due to the cessation of the pumping action of the muscles inducing a momentary stasis of blood in the capillaries, involving a temporary diminution of the venous return to the heart.

It may be remarked that in neither of these explanations is it definitely stated whether the whole of the venous system is regarded as depleted or full—according to one view or the other—or whether the state of *a*, the large venous trunks in the thorax and abdomen, or *b*, the veins among or near the muscles in the trunk and limbs are specially in question. Rapport (108) noted variations in the duration of the secondary rise after moderate and great efforts respectively.

Observations by C. Reid (110) in this Laboratory show that the rate, character and extent of the pressure changes after the end of the exercise vary much in different individuals and in the same individual under different conditions.

The maximum height attained by the subsequent rise of pressure, when such occurs, varies much and bears no precise or constant relation to the maximum height during the period of exertion, though under some conditions it approximates or corresponds to that maximum.

After a type of exercise where the raised pressure during the exercise shows a simple decline afterwards, without a subsequent rise, the rate of the decline varies considerably, and measurements taken at some fixed point of time (e.g., $\frac{1}{2}$ minute) are not to be relied on. Again, in those forms of exercise where a subsequent rise does present itself, occurring in varying degree after a preliminary fall, it is obvious that much will depend on the exact point in the series of changes at which the estimation is made. Measurements at half a minute after the cessation of exertion will naturally give very different results according as a subsequent rise develops or a simple progressive decline occurs; even in the latter type the finding of equal readings at the half minute interval in two different individuals or in the same individual at different times and under different conditions does not prove that the maximum pressures attained during the exercise were equal in the two instances. It is to be emphasised that measurements during the period of exertion constitute the only valid evidence as to the actual rise of pressure. It is not surprising that many discordant results have been recorded by different observers dealing with exercises of different types and duration or even with comparable exercises, when the estimations are made after the end of the period of exertion. Quite small rises (e.g., 16 mm.) have been reported after short spells of severe exertion involving dyspnea with doubling of pulse rate, etc., when the actual pressure during the exercise has really been greatly raised.

Whatever significance may be attached to such estimations for some purposes, it is clear that they are not reliable for determining the

height of the blood pressure response to exertion. The extent and course of this response varies much in different types and degrees of muscular activity—whether the latter be 1, strong or maximal effort with fixation of the thoracic walls, etc., bringing in the factors concerned in Valsalva's experiment; 2, exercises of endurance as in walking, long distance running, cycling, etc.; 3, execution of difficult, though not necessarily strong, movements involving much mental concentration; 4, static contraction of muscles.

A direct relation of the blood pressure rise (associated with exertion) to the amount of work, rather than to its rate, has been affirmed. This is applicable in a general way to certain types of exercise where the mental factor remains tolerably constant, but it is not applicable for comparison between different types involving variable degrees of mental concentration, emotional accompaniments, etc.; in these very different amounts of blood pressure change may be associated with the performance of equivalent amounts of muscular work.

Blood pressures in sleep. That there is a lowered blood pressure during sleep has been found by various observers, often amounting to 15 to 30 mm. at the end of two hours' sleep, then gradually rising toward the time of waking. Such falls of general arterial pressure obviously mean only a relatively limited reduction in the brain vessels —the hydrostatic factor being largely taken off the head vessels in the recumbent posture. Greater reductions have been noted in persons with high pressures in the daytime, e.g., 44 mm. by Brooks and Carroll (22) in hypertonic subjects.

Muller (100) found the systolic pressure to be down to 94 mm. in men and 88 mm. in women during sleep, after a small dose of veronal. In persons with moderate day pressures Blume (15) recorded falls of 15 mm. and 21 mm. in men and women respectively while in subjects with high day pressures the lowering averaged 31 mm. and 39 mm. These observers describe a remarkable constancy of pressures during sleep (rarely more than 5 mm. variation in sleep), even in high pressure cases, in contrast to the great variability seen in the waking pressures. Katsch and Pansdorf (72) while confirming the fall of systolic pressure in sleep—parallel to the depth of the sleep—found that the diastolic pressure sinks little if at all, but on the contrary often rises during the deepest sleep, so that the pulse pressure is diminished. In essential hypertension they observed an abnormal range of systolic lowering; in other hypertensions little or no lowering.

The present writer (88) finds that there are two entirely different

conditions in question in sleep—1, sound sleep with lowering of pressure, 2, disturbed sleep, dreaming, etc., which may be attended by remarkable elevations of pressure, e.g., systolic pressure raised from 125 to 182 mm., or from 130 to 200, etc.; diastolic pressure raised from 75 to 105 mm., etc. These changes were much greater than were induced in the same individuals by moderate exertion (cycling, walking, stair climbing, etc.) straining abdominal efforts, dose of atropin to remove vagus control over the heart, mental excitement, etc. In view of the rapid development of such changes in sleep, especially in dreams of motor effort, nightmares, etc., it is evident that a formidable strain—harmless in the young and healthy person—may thus be thrown on the weak points of the circulatory system, whether these be cardiac with susceptibility to anginal attacks or to ventricular fibrillation and sudden death, or arterial with risk of hemorrhages, cerebral (especially in the recumbent posture), gastro-intestinal or pulmonary. The conception of sleep as a period of quiescence and recuperation has thus to be qualified by the contingency of disturbed sleep with active calls on the nervous system, the heart and the blood-vessels. The mechanism of the rise of pressure in disturbed sleep differs in some respects from that present in ordinary muscular exertion, since in the former the pumping action of working muscles, greatly augmenting the venous return to the heart, is absent. The above-mentioned disturbances may occur during disturbed sleep when there is after awaking no recollection of definite dreaming.

High blood pressure. Notwithstanding the very large amount of attention that the subject has received the causation and mechanism of persistently elevated blood pressure, whether in the form of simple or essential hypertension (the hyperpiesis of Clifford Allbutt (3)) or in association with kidney lesions, remain unexplained. While there is general agreement as to the existence of excessive pressures apart from any recognisable renal lesions and in the absence of any sign of functional inadequacy as tested by the modern methods for estimating renal efficiency, it is also clear from the evidence available that the significance of hypertension is greatly influenced by the co-existence of renal inadequacy, the latter giving a sinister aspect to the condition and seriously altering the prognosis. While there has long been a strong presumption from the clinical side that, 1, toxic substances, probably protein derivatives, are at work, whether *a*, absorbed from the alimentary canal (pressor amines, etc.), or *b*, products of microbic infection, or *c*, abnormal metabolism; or 2, that endocrine derangements

may be concerned (e.g., in hypertension associated with the menopause, etc.) the search for such pressor agents has failed to elucidate the problem, proving almost barren of results. When kidney involvement is also present there are the further undecided possibilities of 3, defective elimination, and 4, the genesis of pressor agents by the damaged renal tissues.

Mosenthal (98) concluded that high or low protein diet does not increase or lower high blood pressures; similarly Newburgh (102) and Squier and Newburgh (118) found high protein feeding ineffective, though acting as a kidney irritant. On the other hand the observations of H. J. Starling (119), bearing on tuberculous cases, indicate a definite elevation of pressure under the continued influence of an abundant meat diet. An important point is raised by the finding of Foster (48) that a reduction of blood pressure under the influence of a continued low protein diet may take two months to develop; this suggests that some negative conclusions with high or low protein diets may possibly be due to periods of insufficient duration being studied. Orr and Innes (105) observed a decided lowering of pressure after the drinking of large quantities of water; they suggested a washing out of metabolites as a probable cause of this effect.

On the other hand Strouse and Kelman (121), examining cases of raised pressure associated with various degrees of renal damage, found that high protein diet caused no rise of blood pressure and that diminution of the protein intake in cases of definite nephritis, while lowering the non-protein N of the blood, did not lower the pressure. Sudden variations of systolic pressure sometimes amounting to 60 mm. were often seen, attributed to emotional causes acting directly on the vasomotor centre; these variations were not affected by alterations in protein intake.

Salt has been surmised to have some relation to high blood pressure and this hypothesis has influenced treatment, as in Allen's regimen with a salt intake cut down to 0.5 gram per diem. The recent work of O'Hare and Walker (103) lends no support to such a view. No relation was found to hold between the blood pressure and the chlorides of blood and plasma, and no effect on the systolic and diastolic levels was seen during wide variations in the amounts (0.5 to 4 grams) of salt taken in high pressure cases without nephritis. Further in subacute nephritis with edema and maximum salt retention comparatively low pressures were often recorded.

Cholesterin has also been suspected, especially by some French

(Chaffard and his school) and Russian investigators. Cantieri's (25) results oppose this idea; he found no relation, in acute or chronic nephritis, between the blood pressure and the cholesterin content of the blood, which in a series of arterio-sclerotic cases was rather below the normal content; also administration of cholesterin does not raise the blood pressure.

Dixon and Halliburton (37) ascertained that the pressor effect of cholesterin given by intravascular injection is negligible.

As regards urea, though high blood percentages of this substance and high blood pressure are often found together, the relation is very variable and it is evident that it is not a causal one. The same statement holds good with regard to the viscosity of the blood, though a group of high pressure cases associated with polycythemia has been recognised.

The search for pressor bodies of endocrine origin (though possibly present in toxemia in pregnancy, etc.) as a cause of persistent high blood pressure has so far proved futile, and the same is to be said with regard to the conceivable possibility of a lack of depressor substances as an operative influence. A similar remark applies to the question of retained pressor bodies when a rise of pressure follows reduction of the kidney tissue below a certain limit, e.g., to one-third, as studied by Passler and Heincke (106), Janeway (70) (with Carrel) and others. It is a remarkable fact that no adequate explanation is available as to how suppression of kidney function kills.

In the presence of structural kidney damage the question of altered function becomes added to that of diminution of functional area. Extracts of kidney have been found by various observers to have pressor effects—Tigerstedt and Bergman's (125) "renin"—and the throwing off of some such pressor agent from disintegrating renal tissue in diseased conditions has been suggested (for some cases of hypertension) by Batty Shaw (12); it has not been found practicable to establish the presence of such agents in the circulation. On the other hand there is the possibility that the whole condition (hyperpiesia) in which persistent high blood pressure is present may be due to toxic agents in the general circulation, secondarily affecting the kidneys and thus leading to an aggravation of the morbid effects.

The latest pressor substance suggested is guanidine, studied by Major and Stephenson (92). These observers observed powerful effects, doubling or tripling of arterial pressure in a few minutes, from intravenous or intramuscular injection of guanidine salts in dogs,—

effects opposed by $CaCl_2$, KCl or NH_4Cl given intravenously. In experimental uranium nephritis (dogs) a marked and persistent diminution in the excretion of guanidine bases was found. In a number of patients with high blood pressures—essential hypertension or with chronic nephritis—a decreased output of guanidine was observed, as compared with the normal daily average of 100 mgm. in normal persons and in patients with normal blood pressure and temperature. It is suggested that kidneys only slightly damaged, e.g., with small vessel sclerosis, might have difficulty in excreting guanidine while other substances might pass and the renal defect fail to be rendered evident by the usual tests till the change has progressed further. With regard to this question evidence is desirable as to the blood pressure in the condition of tetany (e.g., from parathyroid defect) where guanidine has been detected in the blood by Noel Paton (107) and his fellow workers. It would also be of interest to find whether any types of high blood pressure cases are favourably influenced by administration of parathyroid and calcium salts.

The normal relation of blood pressure to the body weight in the healthy state has been shown to be a definite one, as illustrated by the insurance statistics of Symonds and others and by the series of observations of Faber and others, increments of about 10 mm., etc., being found by Symonds in individuals of heavier build at all ages, while the young subjects of Faber showed differences of 6 mm. according to their build. While this appreciable difference holds good in healthy persons the effects of obesity are much more pronounced and have been emphasised by various observers. Among recent investigations, Aubertin's (2) 70 obese subjects (average age 60) showed high pressures in the great majority, only 7 being at or under 150 mm. while 24 non-obese controls of similar age averaged 149 mm. While greater degrees of obesity were associated with higher pressures, arterial sclerosis and chronic nephritis were not found to be the effective connection between obesity and high pressure. Apoplexy and sudden death are evidently related to the high pressures rather than the associations or effects of obesity acting in other ways. It is noteworthy however that Symonds states that fat elderly subjects in good condition and acceptable for insurance commonly have systolic pressures below 140 mm. on an average.

Relation of high pressures to the regulating mechanisms. Marey's Law. Under the circulatory conditions of normal life this law is one that is more honoured in the breach than in the observance. An in-

verse relation of heart rate and arterial pressure only occurs in certain conditions, such as are not usually present. It does not occur in the great majority of normal elevations of blood pressure, e.g., in muscular exercise with its raised pressure and quickened heart, nor in the similar conjunction seen in emotional excitement, nor in sleep where both pressure and heart rate are lowered, nor in some forms of circulatory depression accompanied by a slowed heart and a reduced blood pressure.

A more warrantable statement, much more limited in scope than the so-called law of inverse relation, is that when the blood pressure in the head is raised by an increase of the peripheral resistance in the circulation or by local causes acting on the head (hydrostatic factor, etc.), such pressure tends to increase the controlling power of the vagus centre, provided no other influence plays upon that centre in the direction of reducing its activity—as occurs during motor effort, emotional stress, etc. Conversely a lowered pressure in the head involves diminished activity of the vagus centre unless this is opposed, as may happen, by some concomitant influence tending to stimulate the centre.

It is evident that if persistent high blood pressure is due, as is commonly assumed, to excessive peripheral resistance there must be some agency in action which counteracts the working of Marey's law—since, as is well known, the heart is not slowed even in presence of exceedingly high arterial pressures. Thus in Mannaberg's (93) observations on 241 cases of high pressure, 55 per cent had normal pulse rates, while 43 per cent showed tachycardia and 3 per cent bradycardia; the tachycardias were chiefly in women and probably related to endocrine disturbances (thyroid, etc.). The mechanism of this is unknown. There is no evidence to show why the usual slowing influence of high pressure is not exercised—through direct influence on the vagus centre; and also reflexly through high pressure in the heart and distention of the aortic walls, if such a mechanism exists—as affirmed by Eyster and Hooker (41) for the normal animal, though this view is not supported by the recent work of Anrep and Starling (5) with cross-circulation experiments.

While it is known that high venous pressure acting on the right heart reduces vagus control and accelerates the heart, as Bainbridge found by increasing the volume of the blood, there is no ground for regarding this as a means of abrogating the slowing effect of an excessively high arterial pressure due to abnormally great peripheral

resistance. For when the latter is excessive, e.g., during compression of the aorta at the level of the diaphragm, the right heart (as well as the left) becomes largely distended and the venous pressure very high. But slowing of the cardiac rhythm, due to the arterial pressure, persists in spite of the elevated venous pressure; the arterial pressure dominates the situation, so far as the heart rate is concerned.

As regards the direct relation of blood pressure to the normal functioning of the vasomotor centre, Anrep and Starling have obtained important evidence by a method of cross-circulation. They caused the head of an animal to receive its whole blood supply from a heart-lung preparation while the body of the animal retained its normal blood supply from its own heart; this enabled them to study the direct effects of changes of blood pressure in the head on the medullary centres. They found that a rise of blood pressure in the head actively and almost immediately (after a latency measured in fractions of a second) depresses the activity of the vasomotor centre, causing a fall of blood pressure in the body generally. Changes of pressure in the head induce reverse changes in the body; these are not transitory but last for a long time, generally till the pressure in the brain again changes. Such reversed changes in head and body, first observed by Francois-Franck (49), have been studied by Hedon (60), Tournade, Chabrol and Marchand (127), Foa (47) and others. They have usually been attributed to changes in the heart action through the vagus centre, but such a mode of action is excluded in Anrep and Starling's experiments. It is obvious that a mechanism of this sort must militate strongly against the maintenance of an excessive pressure in the intact circulation.

In view of many facts it is clear that in persistent high pressure in man the condition is not simply one of increased vascular constriction, whether determined by undue activity of the vasomotor centre or by chemical agents acting directly on the walls of the vessels. Simple vascular constriction, raising the general pressure and the pressure in the head would bring into operation various normal regulating mechanisms such as —1, increased control of the heart through direct action of the pressure on the vagus centre together with 2, a direct synergetic inhibiting influence on the vasomotor centre; 3, a reflex depressing influence on the vasomotor centre through (vagus) depressor fibres arising in the aorta and heart, and possibly 4, an alteration of a pressor reflex influence ascending from the terminations of the vagi—a reflex advocated long ago by Pavlov and recently by McDowall.

It is evident that, whatever chemical agencies may be operative in other ways, in persistent high blood pressures there is a marked interference with regulating nervous mechanisms, rendering them ineffective in keeping down the pressure to anything like the normal levels.

The question of a compensatory influence of raised blood pressure. Allbutt regarded high pressure as an attempt of the organism to maintain the equilibrium of the circulation. May the rise of pressure be in some sense compensatory to drive more blood through a vital organ that needs it, e.g., heart muscle or brain or kidney? In the last named the high pressure might conceivably be related to the efforts of the kidneys to excrete concentrated urine, salts or waste products when in excess or when the renal mechanism is inadequate. Possibilities in this direction are suggested by the known existence of the sensitive mechanism by which a defective blood supply to the head promptly sets up a rise in aortic pressure through synergetic changes of increased activity of the vasomotor centre and diminished activity of the vagus centre. A compensatory reaction might conceivably develop in connection with other important organs where the blood supply may be defective from narrowing of arterial channels or diminution in the number of capillaries, or where functioning of the tissue—relatively defective from other causes—might be improved by a higher capillary pressure. A compensatory relation was suggested by Bier with reference to the kidney and later by others. The existence of a compensatory function may be investigated by artificially lowering the pressures (by vaso-dilators, etc.) in order to find whether functional impairment or disturbances, renal, cardiac, or respiratory result from a reduction of the pressure from an elevated level, which, under the conditions present in these cases, had been favourable to efficiency. A recent investigation on such lines by C. Reid (111) does not lend support to the idea of a compensatory relationship as regards renal efficiency, tested by modern methods, blood urea and non-protein nitrogen being estimated and MacLean's urea concentration test, etc. being employed; the raised pressures present, associated with a variety of kidney conditions, were lowered by nitrites, venesection, etc.

As regards the effects of high pressures in causing elongation and tortuosity of arteries, it is obvious that such may result from more than one cause. 1. Impairment of the power of the arterial wall to resist distention may do this, even in the absence of abnormally and persistently high pressures, from the frequent or continued existence of a relaxed condition of the arterial muscle, especially in arteries with

poor support like the temporal, where elongation and tortuosity may develop in an apparently healthy vessel. The present writer (89) has shown that the elongation of an artery by internal pressure is enormously great in a relaxed as compared with a tonically contracted; further, as the process of elongation needs time to develop, the continuous (diastolic) pressure is more effective than the transient systolic rises. Abnormal conditions of the arterial wall may of course diminish its resistance to distention.

2. Apart from such impairment of resistance persistently high pressures tend to elongate the artery and to loosen or pull it away from its normal attachments along its normally straight course, as easily recognised in the case of the brachial, especially in a thin arm, where the vessel is felt as a tube running an elongated and devious course in bold curves down the arm—especially prominent a little above the elbow. The absence of such conditions in the presence of high arterial pressure of unknown duration affords presumptive evidence that the high pressure is not of long standing.

It may be taken as established that high blood pressure readings, when carefully taken, represent approximately correct measurements of the actual intra-arterial pressures as a rule. It is only in a small minority of abnormal cases of thickened arteries with excessive tonic contraction, etc., that serious discrepancy may occur, sclerotic conditions without muscular contraction having no important influence. Digital compression for 3 or 4 minutes or massage of the artery are useful in removing abnormal resistance and have the advantage of not causing congestion of the limb which may arise from repeated compressions by the armlet—with very disturbing results, especially in some susceptible cases, giving erroneous auscultatory indications or actual changes of arterial pressure, etc.

The pronounced effects of mental stress, excitement and worry in producing and maintaining high blood pressure emphasise the significance of the nervous system whether exercised directly through cardiovascular innervation or more indirectly through endocrine or metabolic alterations. The frequent variation of the pressure from day to day or even at shorter periods opposes the idea of structural causation involving increased peripheral resistance and tells against the presence of permanent chemical agencies acting on the vessels directly. The constancy of a lowered pressure during sleep reported in some high pressure cases points in the same direction (Muller, Blume, Katsch and Pansdorf).

Again the strikingly exaggerated pressure changes which may rapidly occur in response to nervous disturbances, emotional causes, etc., as noted by numerous observers in many cases of high blood pressure, bear testimony to the presence of disturbed innervation involving defective regulation as an important factor in the condition. Thus causes of slight elevations of pressure in the normal state may have abnormally great effects in causing rapid and extensive variations in many subjects of high pressure.

Low blood pressure. The mechanism of the acute condition of excessively low pressures seen in circulatory shock, etc.—due to the altered capacity factor dependent on capillary relaxation and later on diminished blood volume—has been elucidated by various investigations, especially by the work of Cannon (24) on traumatic shock and that of Dale with Laidlaw (32) and Richards (33) on the action of the histamine and histamine-like bodies. Similarly the pressure falls in acute infections like cholera, etc., are rendered intelligible. But in persistent low pressures attendant on exhausting diseases or occurring without obvious cause (essential hypotension) the available data are, as in the case of persistent high pressure, inadequate for a satisfactory explanation of the mechanism involved—whether a defective peripheral resistance or defective cardiac output depending, apart from cardiac enfeeblement, or lessened return of blood to the heart as a result of undue expansion of the capacity of the vascular system from capillary or venous relaxation, contraction of venules, diminished volume of blood in circulation, etc. It is also unknown how far such conditions are mediated through the nervous system and how far due to the direct influence of chemical agents—depressor bodies, lack of pressor substances, etc.

As to what constitutes "low pressure" the level below which a pressure is to be regarded as low or abnormal is not sharply defined and no doubt varies considerably, as in the case of high pressure, for the individual and the conditions present. Roughly anything decidedly below 100 systolic or 60 diastolic may be suspected of being "subnormal." Some athletes in good training have such pressures as systolic 105 and diastolic 65.

Subnormal blood pressures naturally exercise a generally depressing influence on the active tissues and tend to establish a vicious circle. There is significance in the observation of Markwalder and Starling (95) that for the mammalian heart (in the heart-lung preparation) an average (innominate artery) pressure of at least 90 mm. Hg is neces-

sary for the due vigour of the cardiac muscle; otherwise the coronary circulation is apt to be insufficient. *Excessively* low pressure injures the nutrition of the heart, favouring enlargement, etc. Muhlberg (99) is quoted by Friedlander to the effect that low pressure after the age of 50, unassociated with any organic lesion to account for it, constitutes the best criterion of life beyond the normal expectancy; it is also stated by Friedlander (50) from Fisher's figures that of 3,389 persons (ages 16 to 60) with systolic pressures of 100 mm. or less there was only 35 per cent of the expected mortality. Symonds (122) considers low pressures after the age of 40 desirable; he also reports that the lowest mortality was found in those subjects who were 15 per cent below the average weight. Pressures not *too* low seem to favour longevity.

The general relation of low blood pressure to tuberculosis has been the subject of several inquiries in recent years. Marfan and Vannieuwenhuyse (94) (700 cases) while finding systolic pressure lowered—more so as the disease was serious or getting worse—do not regard low pressure as excluding improvement or recovery. Normal or raised systolic pressure they regard as a good prognostic. Diastolic pressure falls only in the last stage. These workers emphasise the importance of repeated examinations. De Bloeme (36) in 500 cases by the auscultatory method affirmed the existence of an important group at 100 to 110 mm. S. where the seriousness of the condition was more recognisable by the blood pressure than by other methods, while cases at 80 to 100 mm. were recognisable by ordinary diagnostic means. There seemed to be a general relation between the higher pressures and better conditions of the patients. The most favourable cases of both sexes were between 110 and 150 mm. There was apparently an association between low pressures and the tendency to relapse or the occurrence of relapse, even after the local and general symptoms had subsided; blood pressure rose with improvement.

While regarding blood pressure as being below normal in 60 per cent of early cases, Naucler (101) sometimes found normal or higher pressures in early cases and concluded that low pressure is not a reliable sign of early phthisis. The low pressures seemed to depend more on the severity than on the extent of the disease.

R. J. Cyriac (31) recorded differences in the systolic pressure readings in the two arms in a number of tuberculous cases as E. F. Cyriac (30) did in association with some traumatic conditions.

That excessive smoking can lower blood pressure has long been known and comes into question when systolic pressures at or below 100 mm. are observed.

Pressures in aortic regurgitation. Since the remarkable arm-leg systolic pressure difference was recognised in cases of aortic regurgitation by Hill, Flack and Holtzman (64) in 1909, numerous observations have been made and differences of varying degrees of magnitude have been recorded, one of 200 mm. by Rolleston (114)—leg pressure 350, arm 150—while the differences have as a rule been much smaller. Similar phenomena have been observed in some other conditions—violent muscular exertion in healthy persons, some cases with arterial sclerosis, and in exophthalmic goitre. In these conditions question naturally arises as to which reading represents the "blood-pressure." The mechanism involved has proved difficult of elucidation. L. Hill suggested a different "conductance" in the leg arteries, transmitting the large systolic wave more effectively than in the arm. As the diastolic pressure is virtually if not absolutely similar in arm and leg it is evident that the systolic difference is a phenomenon of wave motion.

There are indications that both *a*, cardiac and *b*, vascular conditions are usually concerned in the mechanism of the arm-leg difference in pressure. That a cardiac factor plays a part is suggested by the clinical evidence to the effect that in man the differential pressure is slight or absent in recent aortic lesions, and is chiefly found in cases of compensated aortic regurgitation with their enlarged heart, large and powerful systolic wave and unusually extensive pulse pressure—conditions also present in greater or less degree in other instances where the arm-leg difference has also been recorded, e.g., exophthalmic goitre, some arterio-sclerotic cases, violent muscular exertion, etc.

In toxic exophthalmic goitre many of the circulatory conditions resembling those associated with aortic regurgitation (large heart, exaggerated pulse pressure, etc.) may be strikingly present. Taussig (123) has observed an arm-leg difference of 37 mm. Hg; the condition has also been described by Harris (59). In a case of arterio-venous aneurysm Lewis and Drury (83) have found that many similar circulatory features were temporarily abolished during artificial closure of the arterio-venous communication, but not the differential pressure, which they attribute to vascular conditions which had become established.

As regards experimental animals there is a conflict of evidence between the results of Bazett who described the immediate appearance of a differential pressure after an aortic valve lesion, and those of Leschke (84) who did not find a differential pressure at this stage.

The important investigation recently published by Bazett (13)

deals with schema results, animal experiments and clinical observations. He concludes that the differential pressure is essentially due to the transference of kinetic energy in a fluid in rapid motion into stress when the flow meets resistance, the relative degrees of slowing thus induced in different vessels and the relative masses of blood concerned being important, while the condition of the arterial wall (suggested by L. Hill) probably plays a part as may also the "breaker formation" of Bramwell and A. V. Hill (20), though not essential. The higher leg pressures are accounted for on these lines, local arterio-sclerotic changes being capable of exaggerating the phenomenon. Larger differences in arm-leg pressure were found with contracted arterioles—involving greater slowing and greater transformation of kinetic energy. It is suggested that the effects obtained by L. Hill and Rowland (65) with warm baths (equalisation of pressures) are explicable in this way as well as on the hypothesis of altered conditions in the arterial walls. The aortic arm-leg difference is thus regarded as a great exaggeration of the normal carotid-femoral difference described by various observers —essentially the water hammer action of Corrigan's "rushing current" in aortic regurgitation.

In animal experiments a reversed differential pressure was sometimes seen, i.e., a pressure higher in the upper than in the lower limb, in association with a forcible heart action with regurgitation present and an apparently low peripheral resistance. No evidence seems to be available of the existence of such a condition clinically.

Capillary pressure. In the attempts to gauge the capillary pressure the various methods and the different criteria applied have produced a discordant and somewhat bewildering assortment of results representing "capillary pressure" as anything between 25 to 50 mm. H_2O and 70 mm. Hg. There are at least four modes of observation that have been used.

1. Blanching methods (skin of finger or hand). Following Von Kries' (75) idea there have been applications by numerous observers, using however different criteria, e.g., the first production of visible paling, used by Basler (11) and by White (132) or complete blanching (v. Basch (10)) or the pressure at which the skin again begins to flush (Recklinghausen (109) and others); much confusion has resulted. Von Basch's figures were 25 to 30 mm. in healthy subjects; he concluded that capillary pressure can vary independently of arterial pressure; Recklinghausen's value was 52.2 mm. Basler with his ochrometer found normal capillary pressures at about 7 mm. Hg, but Landerer

(79) by the same method reported pressures of 17 to 25 mm. Briscoe (21) using Hooker's capsule to cause paling estimated the normal pressure at 23.5 cm. H_2O. Hill and McQueen (66) taking the returning flush as their criterion obtained values of about 10 mm. Hg.

The interpretations put upon the results of blanching experiments naturally depend on the different views held as to the causation of the colour of the skin. There is thus much diversity of opinion as to what is really being measured. While v. Kries, v. Basch, v. Recklinghausen and Basler took the paling of the skin to be due to compression of the capillaries, Lombard (85) and Danzer and Hooker (35) regard the emptying of one or more of the venous plexuses in the dermis as the main cause; they find that paling is not necessarily accompanied by cessation of flow in the capillaries. White (testing the influence of heat, etc.) also concludes that the paling of the skin from external pressure is not an indication of capillary pressure. He obtained values of 4 to 19.5 cm. H_2O. Hill interprets the 10 mm. value (obtained as stated) as indicating arteriolar, not capillary, pressure, *plus* the resistance of the epidermis which has to be deducted. When a capillary area is compressed the internal pressure is regarded as banking up to arteriolar pressure. Such an arteriolar pressure value as 10 mm. Hg is about half the amount reported for capillary pressures by many other observers. The real capillary pressure Hill (62) estimates at something like 20 to 50 mm. H_2O. Observing transparent parts of mammals and frogs by Roy and Graham Brown's (115) method he found such a compressing pressure to cause momentary checking of the blood flow and took this as the true index—as distinguished from a compression stopping the flow, which might require 350 mm. H_2O.

2. Pressure required to cause obliteration of capillaries under the microscope. Lombard (85), who introduced the use of a drop of oil on the skin to permit of direct examination of the capillaries, found very different pressures necessary to obliterate the vessels of different orders. Stated in millimeters H_2O, the following values were obtained —subcapillary venous plexus, 135 to 205; superficial venous branches 205 to 270, most compressible capillaries, 245 to 300, middle-size capillaries, 475 to 545, most resistant capillaries and arterioles, 815 to 950. Methods founded on Lombard's plan have been used by Krauss (74), Basler (11) (capillary tonometer) and Kylin (78). The last named recorded normal pressures of 8.5 to 14 mm. Hg and abnormal ones up to 40 or 50 mm. in glomerulo-nephritis and scarlet fever. Difficulty arises from the unequal compressibility of different capillaries in the field of observation.

3. Pressure required to cause stasis of corpuscular flow under the microscope—by Danzer and Hooker's (35) micro-capillary tonometer, a different criterion from those used in the preceding methods. They found values of 18 to 26.5 mm. Hg averaging 22 mm. Boas and Frant (17) using the same method reported normal capillary pressures at 18 to 22 mm. Hg, rarely above 30 mm.; high pressure cases reading usually between 30 to 60 mm. In essential hypertonus capillary pressure was found to be normal, i.e., below 30 mm. Boas and Mufson (18) found a much higher mortality in a high capillary pressure group (5 deaths in 28). Their post-mortem findings (a small number of cases) did not support Kylin's hypothesis of an association of high capillary pressure with glomerular nephritis.

4. Piercing capillaries with a very fine capillary glass needle (containing saline at a measured pressure) under the microscope to measure the pressure in a capillary loop—by Carrier and Rehberg (26). The values 45 to 75 mm. H_2O in two subjects at 7 cm. below the clavicle—reported by this method—unfortunately unsuited for clinical application—are relatively low and lend support to L. Hill's repeatedly stated view as to the lowness of capillary pressure. The venous pressure was parallel to the capillary pressure.

Supposing that the pressure in the minute vessels of the skin can be accurately measured, there remains the question of the application of such results to the conditions of the general circulation. Pressures in the finger and hand are naturally influenced profoundly by the local conditions of arterial tone as affected by vasomotor influences, heat, cold, exercise, sleep, etc., with the result that the digital pressure may rise while the brachial pressure falls, or vice versa. It is hardly necessary to recall the frequently opposed conditions in the skin and splanchnic areas, as with muscular exercise, asphyxia, adrenalin, etc., also the different incidence of the vaso-dilator effects of acetyl-choline—as described by Reid Hunt (69)—marked in the skin, slight in the liver and intestine, very slight in the voluntary muscles. Again the strong constriction of the renal vessels, which is presumably the cause of the anuria known to occur during short spells of violent muscular exercise, is associated with increased pressure in the skin. The habitually cold or habitually warm hands of different persons, naturally involve wide variations in the pressure relations of the minute cutaneous vessels.

There are thus no means of ascertaining the relations between pressure measurements in the skin and the pressures existing in other parts, internal organs, etc., and how they stand with regard to the

average pressure in the capillary field as a whole, made up as it is of a great and varying distribution of capillary pressure values in multitudinous districts. It is obviously not permissible to speak of capillary pressure in the same sense as arterial pressure, the latter being a definite measurement virtually the same in all the large arteries throughout the body, while capillary pressures vary widely and in different senses in numerous districts under physiological conditions. Still if there is definite association of high readings of pressure in the minute skin vessels in some category of high blood-pressure cases and not in others this—even if not representative of the capillary system as a whole—is obviously a matter of much interest calling for further investigation as to its mechanism and significance. Boas and Mufson report close correspondence in the capillary readings from the same individuals taken many months apart—with some exceptions for which explanations are offered. As to the relations of capillary and venous pressures there is some conflicting evidence. While it has generally been accepted that capillary pressures run much more nearly parallel with venous than with arterial pressures, Boas and Doonieff (16) in a recent investigation (using a needle in a vein connected with a manometer) find that a rise in venous pressure up to 39 cm. H_2O may have no effect on capillary pressure—evidence that the high capillary pressure which may occur in hypertension is not accounted for by high venous pressure. On the other hand, Danzer and Hooker found venous compression to cause increased capillary pressure; Carrier and Rehberg observed a parallelism between venous and capillary pressures. Von Basch and Kraus had formerly emphasised the close relationship between these pressures.

The peripheral resistance. Recent work on the capillary system and the very varied conditions that may obtain in it have re-opened the question as to what the peripheral resistance in the circulation is constituted by. Is the arterial resistance largely supplemented by resistance in the capillary field and possibly also, as suggested by Hooker's (68) work, in the venules, and capable of being altered in an important degree by variations in these as well as in the small arteries?

Favouring the commonly accepted view that the chief resistance is in the small arteries is the greater internal friction depending on the relatively rapid rate of flow in the arteries as compared with the slow flow in the capillaries under ordinary conditions; also such evidence as is available to show that the loss of pressure in passing through the capillaries is relatively small, the great fall from the arterial pressures

ordinarily measured having occurred before the capillary region with its apparently low pressures is reached.

On the other hand there is to be considered the active and strong contractility of capillaries as shown by Krogh (76) and others; Lewis (82) estimated their contractile power as being capable of expelling fluid against a pressure of 50 to 60 mm. Hg, and when contracted, of resisting the entry of fluid up to 90 to 100 mm. Hg. Excessive constriction or closing of an unusually large proportion of the capillary tubes, with diminution of the sectional area of the available capillary bed, must necessarily affect the resistance offered to the outflow from the arterial tree, as well as influencing the capacity of the vascular system, especially in view of the fact that in small capillaries the red corpuscles actually rub against the walls of the tube; it is not simply a matter of internal friction between the layers of the moving blood as in the arteries.

Conditions that might reduce the disparity between the sectional area of the arterial and capillary fields (e.g., closure of many capillaries, arterial dilatation etc.) would naturally tend to enhance in some measure the resistance presented in the capillaries. Again, Lombard estimated the fall of pressure between the small arteries and the veins at 40 to 50 mm. Hg which would postulate a resistance in the capillaries nearly as great as that in the small arteries. But the recent evidence favours low values of capillary pressure, involving a great fall from the pressure in the larger arteries (e.g., 120 mm.) before the capillary field is reached with pressures estimated at one-sixth or one-tenth or even much less—indicating the situation of the main resistance in the circulatory system as being in the small arteries and arterioles.

On the other hand if higher estimates of capillary pressure are correct, especially such as have been reported in some diseased conditions, with a large decline from capillary to venous pressure it is evident that a considerable part of the peripheral resistance must be located in the region of the capillaries and venules. With regard to the possible influence of constriction of the venules, such might obviously have important effects.

Venous pressure. Venous pressures, easily measured in the veins of the arm or hand by the method of Hooker (67), have been found by that observer to be usually between 10 and 20 mm. H_2O, progressively increasing with age from 8 cm. in early youth to 25 cm. in old age. Eyster and Middleton (42) report pressures rarely above 11 cm. normally; Briscoe about the same, 11.4 cm. White (132), using a

method of instantaneous instead of gradual application of external pressure to the vein, recorded lower values, often 4 to 6 cm., sometimes as high as 12.5 cm. Venous pressure has been noted as being raised in nephritic hypertension, in contrast to simple arterio-sclerosis, by Villaret (128) and his associates. But this conclusion is opposed by the results of Leconte and Yacoël (80). Like the pressures in the minute vessels of the skin the venous pressures in a limb are liable to be much influenced by local conditions. And it gives no actual measure of the venous pressure at which the filling of the right heart takes place—the "effective pressure" of Yandell Henderson and Barringer (61)—the difference between the intra-auricular and intra-thoracic pressures. As the latter pressure is variable, this, as Wiggers (133) points out, raises a serious difficulty as regards the application of limb venous pressure measurements to the study of circulatory conditions.

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PERVERTED APPETITES

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The main purpose of this review is to draw the attention of physiologists to a specific form of perverted appetite in cattle, which is of vast economic importance in South Africa, but for which a fairly complete explanation has only been established within the last few years (1), (2). A better title would, therefore, have been "Bovine Osteophagia," although under the wider heading occasion may be taken to refer to current general knowledge on other "perverted appetites." No attempt is made to compile an extensive bibliography; partly because relatively little physiologically clear experimental work seems to have been published, and partly because the literature is scattered through so many diverse journals that much of it is not available in original form in South Africa.

General abnormalities of appetite. The difficulty in discussing perverted appetites in general fashion lies in the difficulty of offering clear explanations of origin, or of physiologial mechanism involved. The *anorexia* or loss of appetite, noted in gastritis, and the *anorexia nervosa* in neurasthenia, passing over into a positive loathing for food in "hysterical apepsia," can hardly be described as perverted appetite. The craving for, and habitual resort to, sedatives, noted in some forms of neurasthenia, is not appetite in the ordinary sense. The *Arsenophagia* deliberately practised by the women of certain mountainous peoples (Hungary, Tyrol) for the sake of their complexions, and by the men for increasing stamina, is hardly perverted appetite although it may become a life-time habit with certain individuals (9).

Bulimia, or increase of appetite, is certainly not abnormal so long as it is a simple demand for increased food, as in convalescence; although it might be described as perverted at the court of the Emperor Nero where decadent diners retired for emesis between courses. The bulimia of diabetes is certainly abnormal but finds superficially rational explanation in the disappearance of unutilised food in glycosuria.

The "craving sensation" common in catarrhal gastritis, temporarily

relieved by eating, and the similar sensation observed in worm infections in children, is commonly attributed to irritation of the intestinal tract, without further attempt to relate clinically observed effect with physiological cause.

Pica. A well defined perversion of appetite or "pica," manifesting itself as an *allotriophagia* or habit of eating miscellaneous indigestible material, is recorded amongst hysterical females during pregnancy, and amongst idiots and the insane (3). Chalk, cinders, slate pencils, string, paper and a variety of similar substances may be introduced into the stomach, but it is not easy to suggest a reason why. It seems an idiotic sort of thing to do and the physiologist is tempted to leave the psychologist to solve the problem.

A similar depraved appetite is a common symptom in chlorosis; apparently manifesting itself as a craving for sour-tasting substances in most cases, but frequently also as a craving for chalk, earth, or other substances which suggest no clue to a rational physiological explanation of the phenomenon.

Amongst animals various unusual appetites may be observed. Occasional grass eating by carnivora is commonly put down by veterinarians to subconscious selection of fibrous material promoting peristaltic intestinal movement. But there are many perverted appetites of definitely serious consequence.

Wool-eating by sheep. Wool-eating by sheep is a habit contracted under a variety of uninvestigated conditions; sometimes credited to faulty dietary but frequently due to the presence of external parasites. In the latter case the wool is simply torn out, and partly swallowed, in an attempt to get rid of the irritation caused by the parasite, and the practice usually disappears after effective dipping. In regard to the former a personal observation may be offered for what it is worth. An experiment upon dietary deficiency in farm stock, carried out in 1916, involved the feeding of penned sheep upon samp (maize endosperm) and a minimal ration of coarse fodder (autoclaved hay); a diet faulty both in mineral and in vitamine content. Most of these sheep developed the wool-eating habit very badly and nibbled at one another continuously, when brought together in the exercise yard. Several died of "impaction" as a direct consequence; post-mortem revealing the rumen literally packed full of wool.

Hutyra and Marek (4) describe wool-eating in Europe as a dietary disorder occurring amongst penned sheep, chiefly during the period of winter feeding; and as occasioning considerable loss of wool, and not

infrequent death through formation of wool-balls in the stomach. The complaint is most commonly noticed amongst lambs, and generally commences amongst a few individuals in the early winter. These gnaw at their mothers' wool, particularly at places soiled by excreta, and others pick up the habit. In how far the habit spreads as a result of the operation of a common cause, and in how far by imitation, is uncertain. The fact that separation of the wool eaters from the rest of the flock assists in checking the development of the habit, suggests that imitation at least plays a part. The perversion is practically unknown amongst sheep at pasture. Hutyra and Marek attribute the disorder to obscure dietary causes, suggesting faulty ratio of the mineral constituents in the food, and particularly a lack of salt; but no definite experiments seem to have been conducted to clear up the etiology of the phenomenon.

Feather-eating. Feather-eating by caged birds and by poultry probably comes into the same category as wool-eating by penned sheep. Klee (7) attributes it to confinement and one-sided nutrition. Others have observed it despite good dietary and attribute it to "sheer boredom" (liebe Langeweile); and to the "vagaries of the female sex," since it is largely confined to hens. In some cases it may commence as a result of parasitic irritation of the skin; in others during passage through the moult. Conclusive experimental work has not been carried out, although the bird is an experimental subject easy to handle.

Hair-licking. The occurrence of hair-balls in the stomach of various animals, arising by licking or biting of their coats, is rarely ascribed to perverted habit, but usually to parasitic infection of the skin; in some cases to natural licking at the time of shedding the winter coat.

Pica in rabies. Allotriophagia of nervous origin may be observed in various animals. In rabies in the dog it is characteristic; customary food being rejected and any sort of alien material within reach, including excreta, being gnawed or consumed.

Coprophagia, or eating of feces, is occasionally observed in animals but generally left unexplained. The literature of dietary deficiency studies reveals frequent references to the development of this habit in the white rat; a habit which may vitiate certain experimental work unless guarded against.

Infantophagia. It is of passing interest to note the most unmaternal trick of eating newly born young, occasionally shown by animals; by carnivores such as the cat, herbivores such as the pig, and omnivores such as the rat. In some cases this appears to arise through fear of

interference with the young, and is most likely to occur within the first few days of birth; in other cases as individual depravity. Most pig-breeders, for instance, are familiar with occasional sows which consume their litter shortly after farrowing. Such sows are generally disposed of, since once the offense has been comitted, there is every likelihood that is will be repeated at subsequent farrowings.

Egg-eating. This habit is known to every fowl-keeper. Once developed, it may become very persistent, although often curable by the simple device of leaving a few blown eggs, filled with mustard and ammonia, in the poultry run. The habit seems to be developed as individual idiosyncrasy, and one culprit may teach others; but it is apparently more widely spread under conditions of faulty nutrition.

Salt hunger. Craving for salt is a phenomenon well known amongst animals, and ranch cattle often develop an indiscriminate habit of consuming any saline material within reach ("pan" deposits). When the available saline material consists, as it often does, of alkaline carbonates instead of sodium chloride, the habit may be regarded as an abnormal appetite rather than a specific hunger.

Mountain climbers and Alpine hunters tell of mountain sheep which show salt hunger so markedly that they press irresistibly in the way of oncoming men, and greedily lick their clothes saturated with saltish perspiration (4).

Drug pica. Certain drugs, notably apomorphin, may produce evanescent pica in animals with feebly developed vomiting center. After an injection of apomorphin the rabbit, for instance, becomes very restless and gnaws anything within reach. Cattle show similar restlessness and may chew at fencing posts, or pick up and gnaw stones and other foreign bodies. This effect soon wears off and can, therefore, not be described as a true pica. There remains the possibility, however, that certain compounds may produce a lasting pica, and in this connection the original theory offered by Ostertag and Zuntz (8) to account for "Lecksucht" of cattle in East Prussia, is of interest.

Lecksucht. The term Lecksucht (licking disease) is used to denote an inclination to lick, gnaw or eat various objects; associated with disturbances of digestion, metabolism, and nervous system. As a clinically recognizable disease it affects cattle and goats almost exclusively, especially young pregnant or lactating cows; sometimes calves and steers; and only very rarely sheep, horses and pigs (4). Lecksucht had been reported from various parts of Germany, and Ostertag and Zuntz (8) state that the soil amelioration measures (Bodenverbesserung)

practised over the East Prussian moorlands at the beginning of the present century, were rendered almost fruitless by the calamitous outbreak of Lecksucht over the reclaimed pastures. Feilitzen (13) reports it from Sweden, and Mosseau in various parts of France.

As described by Ostertag and Zuntz in cattle, the disorder commonly commences with reduced appetite, partially suppressed rumenation, and frequent constipation. The anorexia then passes over into a depraved appetite in which any alien rubbish may ultimately be consumed; mortar, stones, wood, string, clothes, urine, feces. In the initial stages of pica, stalled cattle lick the walls, mangers and neighboring cattle, in casual fashion; but as the complaint progresses the "licking" may go on continually day and night. In the open the affected cattle may eat the toughest grass they can find, twigs of trees and shrubs, and finally soil and rubbish. As Lecksucht develops the animals lose condition, become timid and easily terrified, and finally die of cachexia. In the Erzgebirge (Saxony) the condition is known as "Stallmangel," and in the Black Forest (Baden) is termed "Trockenhinsch" in distinction to "Hinsch" as used to denote osteomalacia; a disease involving the skeletal system, frequently associated with Lecksucht.

The disease is characteristic of particular areas and is normally associated with particular farms, but after bad harvests and in dry years may extend over whole provinces and cause considerable loss. Where the incidence remains low cases generally come under observation in winter, and the gravity then depends upon the duration of the winter. An early spring means early pasture, and cessation of Lecksucht.

In their investigations on the East Prussian moorlands, Ostertag and Zuntz found that sporadic Lecksucht had long been known in the district, occurring chiefly amongst young stock in stalls in which moor hay was used. They could not associate the increased incidence of the disease with the recent reclamation processes as such (drainage, cultivation) but rather concluded that it was due to a wider use of moorland hay. They then conducted experiments on calves, fed in small batches upon various samples of hay, and came to the conclusions:

1. That the moor hay of the Johannesburger Heide definitely produced fatal Lecksucht in as short a time as 10 to 15 weeks.

2. That the severity of Lecksucht varied according to season and that hygienic factors also entered; development being less rapid in warm, well-lighted stalls.

3. That horses could be fed, without bad effect, on the hay which produced Lecksucht in cattle.

4. That hay cut before flowering was harmless, but later cuttings harmful. Aftermath was also relatively harmless; as also clover hay grown on the same soil, and moor hay manured with nitrate of soda.

5. Steaming, or conversion into "Braunheu," provided this was prepared under conditions which allowed of proper self-heating, removed the injurious influence.

6. The disease did not occur amongst animals naturally grazing over the moors (selective feeding?) and cattle recovered when turned out to pasture. Medicaments, and use of supplementary concentrated foods, did no good; nor did a supplement of sodium chloride and potassium phosphate.

Analyses of the hay were carried out and the possible significance of the relative proportions of mineral elements considered. The final conclusion arrived at, chiefly on the evidence of the steaming and fermentation experiments, was that the disease could be best explained as a "cumulative poisoning" effecting disturbance of metabolism, particularly of blood and bone; possibly due to more than one poison since the aftermath produced mild pica but not the emaciation characteristic of the later stages of Lecksucht.

The experimental data of Ostertag and Zuntz, however, were far from conclusive and Marek considers that Lecksucht has no single origin but is due to a variety of factors in which "alkali deficiency" plays the most important rôle. Zuntz himself later tried to explain the disease as due to excessive preponderance of potassium over sodium in the hay. Ibele (6) also lays stress upon the alkali content of hays incriminated in Lecksucht and Osteomalacia; not so much upon the chlorides as upon the organic salts of sodium and potassium. Marek (5) considers that the absolute content of sodium and potassium is too low and the ratio of alkali to acids (Cl, SO_4) so unfavorable that physiological deficiency of alkali results; and points out that the "alkali-alkalizität" (i.e., preponderence of milligram equivalents of sodium and potassium over milligram equivalents of chloride and sulphate) in Lecksucht hays is below 17, or even negative. He also considers that high phosphorus content in proportion to base may indirectly impose a drain upon the sodium and potassium reserves of the body, and so again lead to "alkali deficiency." In a later paper (1924) Marek gives his views upon mineral metabolism generally, in relation to the etiology of bone diseases, but in spite of his high reputation as a veterinary clinician, they will not

find extensive acceptance amongst physiologists and biochemists. Experiments are now in progress at Onderstepoort (Division of Veterinary Research, Pretoria) in which it is hoped to clear up many points in regard to mineral metabolism of cattle and sheep, but existing data in the American literature seem already sufficient to demonstrate the untenability of Marek's views. The etiology of Lecksucht is, therefore, still obscure, and awaits more thorough experimental study.

Thomasmehlseuche. This name is given to a form of Lecksucht occurring on poor soils manured with basic slag in the Oldenburg district in Germany. Popp (10) reports unusual hematological features and successful treatment by intravenous injection of "Humalcal," a high-priced proprietary preparation consisting of the soluble calcium salt of an easily oxidisable organic acid ("Humalsaüre") of undetermined constitution. Ibele recommends change of diet and inclusion of alkali-rich foods. Neumann and Reinhardt (11) attribute it to a disturbance of mineral metabolism induced by vitamine deficiency. No really conclusive experimental work has been yet carried out.

Bovine osteophagia. Of all the "perverted appetites" of animals, *osteophagia* in South African cattle has received the most detailed attention, and is susceptible of the most complete explanation. Until 1919, it was referred to by the more general term, pica, but now that its significance is fully understood in relation to the etiology of the cattle diseases "Lamsiekte" (Theiler and co-workers, 1920), and "Styfsiekte" (publication pending), and to the nutritional problems presented by aphosphorosis in stock (Theiler, Green and du Toit, 1924), the specific term osteophagia, is used. The special predilection for bones is quite definite, and has been conclusively shown to be a symptom of phosphorus deficiency in the animal.

Depraved appetite in cattle has been recognized in South Africa as far back as the records of the sub-continent go. In view of the fact that its full significance has only been so recently recognised, a quotation (referring to Namaqualand) from Le Vaillant's "Travels into the Interior Parts of Africa in the years 1780–1785" (14) is of singular interest:

> . . . . quality of the herbs may be easily distinguished, and I employed a particular method to discover it. After I had arrived in any canton, I judged of the harshness of the grass upon which they (cattle) had been fed, by their dispersing themselves all over my camp and eagerly searching for the bones left by my dogs, in order to ease their teeth. As the bones were of a calcareous nature, by gnawing them they blunted the force of that irritation and acidity which tormented them. We, therefore, never threw our bones into the fire. When these

were wanting, dry wood or stones supplied their place; and when they could not find these resources they even gnawed each others' horns. When the pastures were good this ceremony never took place.

Written a century and a half ago, this description of osteophagia is as perfect as if written today, and it seems amazing that the correct explanation should take so long to arrive. For the phrase "irritation and acidity," substitute the word "aphosphorosis," and the gap between 1785 and 1925 is bridged.

In the intervening years osteophagia, in varying degree, has been so widely recognized over the whole country that most farmers have failed to treat it as a serious abnormality, look upon it as a natural thing, and only remark upon it when it is so pronounced as to constitute a public nuisance, i.e., when it passes over into allotriophagia and the cattle congregate around the homestead or Kaffir huts and devour miscellaneous rubbish; cinders, bags, riems, condensed milk tins, and the "weekly washing waving in the wind." A perfectly good shirt, chewed from the drying rope, attracts attention, and such acute manifestations of depraved appetite do not escape notice; but a man brought up in districts where allotriophagia is common may quite ignore the *subdued craving* manifested only for *bones*, displayed by cattle on farms where pica is comparatively mild.

That some connection existed between pica and the devastating disease *Lamsiekte* (lame-sickness) had been surmised for many years, but the fact that depraved appetite was so widespread over the whole country, while Lamsiekte was often so strictly localised, created the impression that the correlation was fortuitous rather than essential. In 1919, however, the discovery of the true etiology of Lamsiekte at once showed that although osteophagia occurred independently of Lamsiekte, Lamsiekte did not occur independently of osteophagia. Lamsiekte itself was then shown to be a direct intoxication due to ingestion by the grazing cattle of carcass debris infected with a specific anaerobic toxicogenic saprophyte, now designated *Parabotulinus bovis*. Such debris is common on ranching areas where land is cheap but labor scarce, and carcasses (including wild animals, game, birds, tortoises, lizards) are allowed to rot undisturbed. The point is that decaying skeletal debris is left untouched by normal cattle and only eaten when depraved appetite is shown; hence osteophagia is a necessary precursor of Lamsiekte, which does not occur if depraved appetite is prevented.

Owing to the enormous losses from Lamsiekte, osteophagia itself came in for special investigation and was soon found to be directly due to

phosphorus deficiency in the natural veld grazing. Study of the various degrees of depraved appetite showed that in its mild form the craving is a highly specific one, manifested only for bones, although in its aggravated form all sense of discrimination is lost. A "mild craver" is fastidious in its selection of clean weathered bones, but an "extreme craver" has even been observed crunching a living tortoise with the blood dripping from its jaws; or eating pieces of hide with adherent putrefying flesh.

The posture of osteophagia is characteristic and can be recognised at long distances; head tilted in the air, jaws grinding steadily on the discovered bone. A group of bone eaters may provide a veritable concert.

Recognition of the specificity of craving led to the introduction, for experimental purposes, of a simple method of expressing both the degree and prevalence of osteophagia on a definite chartable basis. Bones at various stages of putrefaction were offered in troughs and the behavior of the cattle noted. It was found quite easy to pick "stinking bones." which only the worst cravers would touch, and perfectly sweet bleached bones which even the mildest craver would at least toy with. Intermediate degrees of perverted appetite could be identified by intermediate bones, but for routine testing two grades were found sufficient; "sweet or bleached" and "distinctly rotten." The latter were picked out as "veld bones" which would produce Lamsiekte if dosed in sufficient amount, but were then autoclaved to render them non-toxic (toxin thermolabile). The former were selected so as to be quite unobjectionable to any beast which showed any clearly definable craving at all.

Data were then accumulated in regard to the prevalence of osteophagia throughout the Union of South Africa; correlation with composition of soil and vegetation; seasonal incidence; relation to age and sex of animal; cure and prevention; and various other factors. The general results may be summarized as follows:

1. Osteophagia is definitely due to phosphorus deficiency and to nothing else. Administration of any phosphorus compound utilizable by the animal, leads to rapid disappearance of the perverted appetite; within a few weeks, if the supply is large enough. Bran, bone meal, precipitated calcium phosphate, sodium phosphate, are all effective; even phosphoric acid itself. The efficacy of this last compound disposes of the possibility of any other mineral deficiency being involved under ordinary circumstances. Simple salts containing calcium, magnesium, sodium, potassium, iron, chloride and sulphate, have no effect in alle-

viating pica; indeed, carbonates of potassium and calcium aggravate the craving. Osteophagia can be produced in stalled cattle on artificial rations so selected as to reproduce the phosphorus intake of natural veld conditions, but the process is lengthy and uncertain unless animals are selected which have already shown osteophagia at some time in their past history.

2. Owing to the widespread deficiency of phosphorus in South African soils, osteophagia is prevalent in varying degree all over the Union. Its incidence can be definitely correlated with the percentage of phosphorus in the natural vegetation. The gross quantity of vegetation eaten of course influences the total phosphorus intake, and the phosphorus content has, therefore, to be considered in relation to the general feeding value of the grass. As a broad generalization, osteophagia may be regarded as developing when the "phosphorus: energy ratio" falls to about 0.6 Phosphoric Oxide per 100 Starch Equivalent. On poor winter grazing of less than 0.1 per cent phosphoric oxide (on the dry matter) osteophagia is very pronounced.

3. In typically phosphorus-deficient areas 90 per cent, or even more, of the grazing cattle may show osteophagia, and the percentage of cattle showing it in any given herd is a useful (though not infallible) guide to the prevailing degree of phosphorus deficiency in the vegetation. A small percentage of cattle in any given herd may never develop osteophagia at all, in spite of the fact that nutritional aphosphorosis can be demonstrated by response, in weight and condition, to phosphorus feeding. A small percentage (chronic cravers) also fail to lose the perverted appetite even when dosed with bone meal in large excess of their physiological requirements.

4. Cattle showing osteophagia improve enormously in condition when phosphorus deficiency is rectified. In cows the milk yield is much increased. In young growing stock the rate of increase in live-weight may be doubled or trebled by a small ration of bone meal. The disease Styfsiekte (probably the so-called "cripples" of New Zealand, Australia, and Texas) is in all probability a straight aphosphorosis. Cattle improving in weight and condition, on a phosphorus supplement, do so partly in virtue of greater food consumption (improved appetite) and partly in virtue of better utilization of food per unit consumed.

5. Horses do not show osteophagia when exposed to the same veld conditions as cattle. Sheep either do not show it at all, or show it in mild timid form difficult to detect; although feeding experiments may

reveal the need for phosphorus. Goats may show it in irregular spasmodic fashion.

6. With cattle, the amount of phosphatic supplement required to prevent osteophagia is highest for breeding cows at the height of lactation; least for old oxen; and intermediate for growing stock. Experiments in which a mixed herd was maintained in "osteophagia equilibrium" by testing for craving each week, and dosing just sufficient bone meal to prevent continuous osteophagia, gave the following figures for phosphorus requirements on a typical Lamsiekte farm (Armoedsvlakte, Vryburg, C. P.); oxen ½ to 1 ounce of bone meal per head per day; growing stock 1 to 2 ounces; cows 2 ounces rising to 6 ounces at the height of lactation. Quantities vary with soil, vegetation, and season. The optimum quantities required to secure maximum growth and condition are somewhat higher than those required for bare prevention of osteophagia, and consequent protection against Lamsiekte.

7. Osteophagia varies with the season of the year. The very young grass of early spring generally contains, for a short period, sufficient phosphorus for normal nutrition. As the grass matures, carbohydrate formation in the plant proceeds so much faster than phosphorus absorption from the soil, that the percentage of phosphorus in the grass rapidly falls. After the seed has fallen the phosphorus content of the standing hay may be so low that osteophagia becomes universal in the grazing herd. On the farm "Armoedsvlakte" in 1919 the first green blades showed 0.5 per cent phosphoric oxide, expressed on the dry matter, and so long as the cattle got sufficient of this very young grass, osteophagia was practically absent. The winter grazing showed only 0.08 per cent or about one fifth of that in a European meadow hay, and during the long rainless autumn and winter (characteristic of the area) the extent of osteophagia was over 80 per cent of the grazing herd. The curve plotted throughout the year for phosphorus in the grass, closely reflected the curve showing prevalence of osteophagia.

Although such seasonal variation can be demonstrated on pasture from which the old grass of the preceding year has been removed, the actual prevalence of osteophagia in ordinary ranching practice is usually extensive the whole year round. In the areas of cheap arid land chiefly concerned, no winter feed is grown, and the wasteful practice of "veld-burning" is adopted to clear the way for the new seasons' growth. Since, however, sufficient old grass must always be left to tide the animals over until the young grass coming up over the "burns" responds to the late rains, and the phosphorus content of the new grass rapidly

diminishes as it becomes more luxuriant, the net result is that the pasture as a whole is phosphorus-deficient all the year round; and osteophagia abates for a month or two at most; then only in oxen and growing stock, and rarely in lactating cows.

Psychology of osteophagia. It certainly seems a remarkable manifestation of instinct or intelligence that physiological deficiency of phosphorus in cattle should be reflected by a specific craving for bones; the only accidentally available source of phosphorus capable of relieving the craving; though a source which, if permeated with Lamsiekte toxin, is fraught with a danger greater than aphosphorosis. Precipitated calcium phosphate, or other unfamiliar source of utilizable phosphorus, serves the same purpose, but if displayed in troughs no craving is shown for it. The predilection is for bones, and in mild osteophagia is for the most tasteless and odorless bones the cattle can find. With the majority of semi-wild ranch-bred animals the craving can be removed in a few weeks, and restored as rapidly, by the simple process of supplying or withholding a bone meal ration. A small proportion of such cattle never develop the craving in spite of the fact that they show the malnutrition characteristic of aphosphorosis; a small proportion retain the habit despite forced feeding on large quantities of bone meal; but the great majority react with astonishing delicacy. On the other hand, cattle reared under artificial supply of phosphorus-rich foodstuffs, and which have, therefore, no past history of osteophagia, only acquire the craving with difficulty when fed upon phosphorus deficient rations under stall conditions. Once an animal has acquired the habit it can usually be removed from its native area as a good subject for study of osteophagia, but even then the response to supply and abstraction of phosphorus is less reliable than it is on the natural veld.

The simplest explanation is that certain members of a grazing herd, on the verge of allotriophagia, or indiscriminate craving, develop a selective taste for material which happens to be available, and at the same time effective in creating a feeling of well-being; that they learn by experience that certain recognizable available objects offer more relief in the long run than others; and that other cattle imitate the procedure as a "trick of the herd" but soon discover that it satisfies an instinctive need. An objection to this view is that young cattle brought up in an area carefully cleaned of all bone debris have yet been found to manifest osteophagia the first time bones were displayed before them. It is possible that the cleaning process may not have been perfect,

but so far as is known these individuals had no chance of learning the habit, i.e., of profiting by the experience of an osteophagic herd.

This question of what one may term the "psychology of osteophagia" remains to be investigated as opportunity allows, but in whatever manner osteophagia does spread through a herd the fact remains that, once it is developed, the cattle are very persistent in their search for bones; may spend a considerable portion of their precious grazing time, on a sparsely covered veld, in snuffling under grass tufts, and so indeed succeed in finding fragments undetected by the eye of even a careful human observer.

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THE PHYSICAL CHEMISTRY OF THE PROTEINS

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TABLE OF CONTENTS

I. Introduction 350
Classification of proteins 350
Composition of proteins 351
Structure of proteins 353
Dimensions of proteins 354
II. The molecular weights of the proteins 355
Molecular weights estimated by measurements of osmotic pressure 356
Molecular dimensions estimated by ultrafiltration 357
Minimal molecular weights estimated from combining weights ... 357
Minimal molecular weights estimated from containing weights .. 358
Probable molecular weights of the proteins 360
III. The relation between the basic amino acids in proteins and their acid combining capacity 361
Basic amino acids in certain proteins 362
Acid combining capacity of certain proteins 363
IV. The relation between the dicarboxylic acids in proteins and their base combining capacity 372
Dicarboxylic acids in certain proteins 372
Base combining capacity of certain proteins 374
V. The number of acid and of base combining groups in proteins 376
VI. The titration of the acid and base combining groups in proteins 378
VII. The dissociation of the acid and base combining groups in proteins .. 385
Dissociation of weak uni-univalent electrolytes 386
Dissociation of weak polyvalent electrolytes 388
Apparent dissociation of proteins 390
VIII. The ionic mobility of proteins 391
Conductance method 392
Hittorf method 393
Moving boundary method 393
IX. The isoelectric points of proteins 395
Isoelectric points of water soluble proteins 397
Isoelectric points of salt soluble proteins 399
Isoelectric points of slightly soluble proteins 400
X. The solubility of proteins in water 402

XI. The solubility of proteins in dilute acid, base or salt solutions..... 406
Solubility in systems in which proteins are the saturating bodies 406
Solubility in systems in which protein compounds are the saturating bodies..... 407
XII. The solubility of proteins in concentrated salt solutions..... 410
Effect of the protein concentration upon solubility..... 410
Effect of the salt concentration upon solubility..... 413
Effect of the hydrogen ion concentration upon solubility..... 415
Significance of the equation defining solubility in strong salt solutions..... 418
XIII. The viscosity of protein solutions..... 419
Relation between the physicochemical state of a protein solution and its viscosity..... 419
Relation between the concentration of a protein solution and its viscosity..... 421
Relation between the viscosity of a solution containing several proteins and the viscosities of the several proteins..... 423
Relation between the molecular weights of proteins and the viscosities of their solutions..... 426
XIV. Bibliography..... 428

I. Introduction. The physical chemistry of the proteins must in the last analysis depend upon their structure. The composition of proteins is revealed by analytical chemistry, and the behavior of proteins by physical chemistry. The simultaneous consideration of the results of these diverse experimental methods is supplanting the empirical, descriptive chemistry of the proteins with a theoretical chemistry.

Classification of proteins. The descriptive chemistry, the analytical chemistry, and the physical chemistry of the proteins have developed almost independently of each other. Descriptive chemistry had its origin in the observations of those investigators of the eighteenth and nineteenth centuries who sought methods for the extraction of proteins from different vegetable and animal sources. Methods of extraction were followed by methods of separation and purification. Water, acid and alkali were the first solvents to be employed, and to these alcohol and neutral salt solutions had been added by the middle of the nineteenth century. The water-soluble, heat-coagulable proteins that are called albumins, the alcohol-soluble proteins that are called prolamins, and the class of substances that are dissolved by neutral salts and that are called globulins, had all been discovered by 1859 (243).

In 1859 Denis published his classical "Mémoire sur le sang . . . . suivi d'une notice sur l'application de la méthode d'experimentation par les sels a l'étude des substances albuminoides" (71). In it he

described the behavior of globulins and also a method for the separation of proteins by means of the solvent action of neutral salts. His contemporaries, Panum (256), Claude Bernard (20), and Virchow (350) observed the precipitating action of neutral salts, and evolved methods for the separation of proteins by this means. The diverse character of proteins from different sources thus began to be recognized and to supplant the notion of Beccari and of Liebig that proteins differed in state and not in substance (243). The solvents that have been employed in extraction, and the precipitants that have been effective in separation have thus given rise to a technique for preparation and to a classification of proteins.

Composition of proteins. The elementary compositions of different proteins are so nearly alike as to have led mid-nineteenth century chemists to believe that the same proteins were present in animals and vegetables (179), (230). In 1842 Dumas (75) demonstrated differences in the elementary composition of the proteins by means of his newly developed method for the determination of nitrogen, and thus disposed of Liebig's notion of their identity. The elementary analyses of the main protein constituents are, however, so uniform that it is impossible to ascribe to them observed differences in the behavior of the proteins. Differences in the solubility of specific proteins, upon which their classification depends, cannot be ascribed even to their rare elementary constituents.

Proteins are, however, composed of nitrogen-containing substances known as amino acids, and differences in behavior may often be ascribed to differences in the distribution of these constituents of the protein molecule.

The first of the amino acids were discovered a little over a century ago.[1] Leucine and glycine were isolated from proteins in 1819 and 1820 by Proust (286) and by Braconnot (29). Though cystine had previously been described by Wollaston in 1810 (363), it was not isolated from a protein until 80 years later (165), (228). With the exception of tyrosine, which was found by Liebig in 1846 (180), no new amino acid was isolated from a protein for 45 years, or until 1865. In 1864 only three amino acids had thus been recognized among the decomposition products of proteins; and in 1904 seventeen amino acids were known. In the intervening 40-year period, a new amino acid was dis-

[1] For the history of the discovery of the amino acids, reference may be made to the Biochemisches Handlexikon (2), and Plimmer (281).

covered every few years and the main constituents of the protein molecule were recognized. Although 20 years have elapsed since 1904, a period one-half as long as that which saw the discovery of 15 new amino acids, but few additions to our knowledge have been made. In 1907 Abderhalden and Kempe (4) announced a new amino acid, oxytryptophane, whose structure has recently been reconsidered (5). Oxytryptophane has not yet been reported by other investigators.

TABLE 1

The isolation of the amino acids from protein hydrolysates

DATE	AMINO ACID	ISOLATED FROM PROTEIN BY	
1819	Leucine	Proust	(286)
1820	Glycine	Braconnot	(29)
1846	Tyrosine	Liebig	(180)
1865	Serine	Cramer	(60)
1866	Glutamic acid	Ritthausen	(292)
1869	Aspartic acid*	Ritthausen (293); Kreussler	(161)
1875	Alanine	Schützenberger and Bourgeois	(310)
1881	Phenylalanine	Schulze and Barbieri	(308)
1889	Lysine	Drechsel	(73)
1890	Cystine**	Külz (165); Mörner	(228)
1895	Arginine***	Hedin	(114)
1896	Histidine	Kossel	(158)
1901	Proline	Fischer	(81)
1901	Valine****	Fischer	(81)
1901	Tryptophane	Hopkins and Cole	(139)
1902	Oxyproline	Fischer	(82)
1903	Isoleucine	Ehrlich	(77)
1907	Oxytryptophane	Abderhalden and Kempe	(4)
1918	β-hydroxyglutamic acid	Dakin	(62)

* Previously discovered by Plisson (283) in 1827.

** Previously discovered by Wollaston (363) in 1810.

*** Previously discovered by Schulze and Steiger (309) in 1886.

**** Previously discovered by v. Gorup-Besanez (88) in 1856.

In 1918, Dakin described β-hydroxyglutamic acid, and later showed that it was present in relatively large amounts in certain proteins (62), (63), (64), (65). Since then certain new components of the protein molecule have been reported (307), (343), (87), (229), but these have not yet been isolated in large amounts, and like oxytryptophane, have not yet been studied in more than one laboratory.

In table 1 the amino acids have been arranged in the order of their discovery, and this relation has been graphically represented in figure 1,

by plotting the number of amino acids that have been isolated from a protein source as ordinates, and the year of their discovery as abscissae. That the rate of discovery of the amino acids has decreased, despite the continued researches of investigators who had previously succeeded in discovering new amino acids, suggests that the period of discovery nears its end. The fact that the percentage composition of certain proteins (62), (64), (65) is now extremely well known leads to the same conclusion.

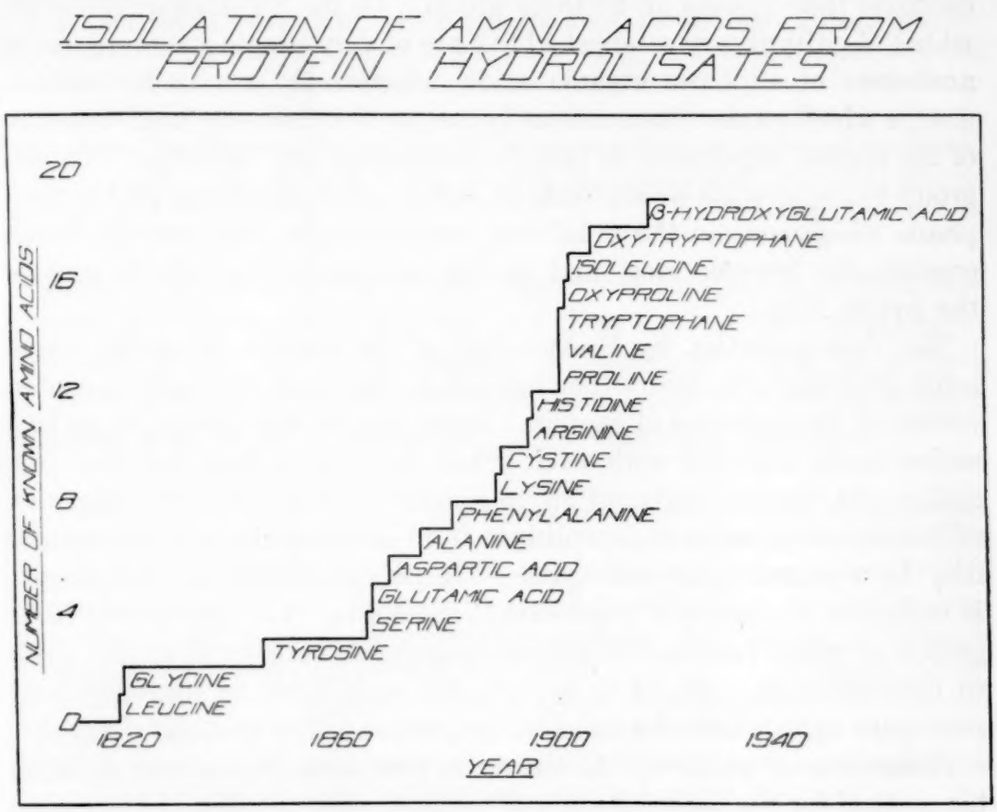


Fig. 1

Structure of proteins. The discovery of the amino acids was followed by the study of their composition, their structure, and of methods for their quantitative estimation, and upon this knowledge rests the composition and structure of the proteins. Amino acids contain both acid and basic groups. The acid groups are identical with those possessed by organic acids, and the basic amino groups have as their simplest prototypes aliphatic amines. Certain of the amino acids possess more than one basic group, or more than one acid group. Two of these, arginine and lysine, contain two amino groups and one carboxyl group,

while a third, histidine, contains one amino group and the basic imidazol ring. These three amino acids thus contain more than one basic group; and three others, aspartic, glutamic and β-hydroxyglutamic acids, are monoamino dicarboxylic acids. In the former basic properties and in the latter acidic properties predominate.

Certain of the other amino acids possess groups which may render them dibasic acids. Such hydroxyacids as tyrosine and serine may possess acid properties (90) (107), since in addition to one amino and one carboxyl they possess an hydroxy group. Of the nineteen amino acids whose identity is now established five are of very simple structure, being analogues of aliphatic organic acids, whereas the remainder contain groups which render them unique in the protein molecule, and therefore of the utmost importance in protein metabolism and behavior. To this group belong cystine which contains sulfur, oxytryptophane and tryptophane which contain the indol ring, phenylalanine and tyrosine which contain the benzene ring, and proline and oxyproline which contain the pyrrol ring.

The demonstration by Fischer (83) of the manner in which amino acids combine with each other has led to the most generally accepted notion of the structure of protein. According to this notion, when two amino acids combine with each other to form a dipeptid, one free amino and one free carboxyl group remain. These may combine with still other amino acids or dipeptids to form polypeptids, and this process may be repeated again and again. The polypeptid linkage thus results in molecules consisting of combined amino acids. The free acid or basic groups of which polypeptids and proteins are still possessed enable them to combine with acids or bases, or with each other to form the vast molecules upon which the colloidal properties of the proteins depend.

Dimensions of proteins. At the same time that Dumas was devising his method for the determination of nitrogen, and the first of the amino acids were being discovered, Graham was distinguishing between crystalloids and colloids (89). He classified as colloids substances that were unable to penetrate certain membranes, that diffused very slowly and that could not be crystallized. The first two of these definitions apply to most proteins and the last to a few. Egg and serum albumin may be crystallized from half saturated ammonium sulfate solutions by the method of Hofmeister (138), as variously modified by Hopkins and Pinkus (140) and by Sørensen and Høyrup (322). Certain of the hemocyanins have also been crystallized by this method (124), (287). Hemoglobin, Bence-Jones' protein, (74), (80), (115), (142), and many

of the vegetable globulins may be crystallized from their solutions (243). On the other hand, the globulins of serum have never been crystallized, nor has casein, gelatin, nor the prolamins. Proteins vary thus in their crystallizability. A distinction between proteins that crystallize and other colloids was recognized by Picton and Linder as early as 1892 In the first of their papers upon "Solution and Pseudosolution," they remark,

> In seeking for a body likely to be of service in connecting colloidal with crystalloidal solution, we naturally turned our attention to the case of haemoglobin. This substance is easily obtained; it forms several crystallisable derivatives, and it affords an instance of enormous molecular complexity. . . . . Obviously it is of great interest to determine the analogies between a crystallisable solution containing these exceedingly complex molecules, and the colloidal solutions, some at least of which we have seen contain aggregates large enough to scatter and polarise light (279, p. 157).

Because of the large size of their molecules proteins have been considered as colloids, and laws that had been derived for aggregates of identical smaller molecules, usually of inorganic origin, have been applied to them. Certain distinctions must, however, be made between colloidal aggregates of matter and large organized molecules.

> If a metal or a simple compound is brought to the colloid state, the dispersed particles are always aggregates of simpler but similar smaller portions. If, on the other hand, the particles are those of a non-associated substance of high molecular weight, of the same degree of dispersity as the above, we have particles formed of a combination of several heterogeneous groups of atoms (for instance, albumin, which is formed of various amino acids). . . . . At first sight it appears that the second example is more complex than the case of the colloidal metal; but one must not overlook the fact that the various groups which build up the complex compound often retain their characteristic properties, and stamp the whole molecule with their physico-chemical behaviour (263, p. 8).

The colloidal dimensions of protein molecules may be inferred from their capacity to scatter light. This property is responsible for the so-called Tyndall phenomenon, and indicates that the molecules are large in comparison with the wave-length of light. A large number of other properties, such as the high surface tension (235) and the high viscosity (195), (303) of protein solutions have also been ascribed to their size.

II. The molecular weights of the proteins. It is upon the large molecular dimensions of the proteins that such colloidal aspects of their behavior depend, as their failure to pass through many natural

and artificial membranes. The failure of proteins to pass through membranes has led to two methods of estimating their molecular weights.

Molecular weights estimated by measurements of osmotic pressure. The first depends upon the osmotic pressure produced by protein solutions. Such pressures should be a function of the number of dissolved molecules and ions (348). The application of this simple principle to proteins is, however, rendered difficult by their reactivity and multivalent character. The osmotic pressure produced by solutions of uncombined protein is smaller than that produced by protein compounds[2] (181) (191), (294). The permeability of membranes to the inorganic ions into which protein compounds dissociate, and their impermeability to the proteins, gives rise to complicated membrane equilibria. Equilibria of this kind have been extensively studied by Donnan (72) and by Loeb (195), and have been the subject of a previous article in PHYSIOLOGICAL REVIEWS (135).

Estimates of the molecular weight of hemoglobin, made from osmotic pressure measurements upon solutions containing electrolytes, have varied between 15,000 and 32,000 (149), (294), whereas recrystallized solutions of hemoglobin exhibited a lower pressure, and therefore a higher molecular weight, approximately 48,000 (290). The osmotic pressure of "ash-free" gelatin led Smith (315) to postulate a molecular weight of 96,000 for this protein. The molecular weights of these proteins may be higher than these estimates, since the measurements upon which they depend were not corrected for the Donnan equilibrium, but they can scarcely be lower.

Sørensen was the first investigator to correct his osmotic pressure measurements for the Donnan equilibrium (320). The estimates of protein molecular weights made by earlier workers all prove to have been too small because this correction was not made (58). Sørensen and his collaborators have studied three proteins, egg albumin (320), serum albumin,[3] and serum globulin.[3] They have estimated that the molecular weights of the first two are 34,000 and 45,000 respectively. The molecular weight of serum globulin varied in their experiments between 80,000 and 140,000, depending upon the amount of phosphorus in the globulin preparation. Since euglobulin is the fraction rich in

[2] Many properties of the proteins that depend upon osmotic phenomena, such as swelling (284), (285), have not been considered in this review. They have recently been summarized by Loeb (195).

[3] Unpublished data.

phosphorus, the higher value presumably reveals its molecular weight and the lower, that of pseudoglobulin.

Theoretically it should be possible to determine molecular weights not only by their osmotic pressures, but also by vapor pressure, freezing point, and boiling point measurements. These methods are applicable only to soluble proteins, and have thus far not been applied with great success. The boiling point method cannot be used in the study of most protein solutions, since they are altered by high temperatures. Freezing point determinations have been made upon protein solutions from time to time (37), (97), (299), (337). The results of these related methods are rarely unequivocal, since they record the total number of dissolved molecules and ions. The slightest trace of impurity, or of compound formation, leads to larger changes than are due to the protein molecules, and therefore to too small estimates of the molecular weight.

Molecular dimensions estimated by ultrafiltration. The failure of proteins to pass through membranes may be employed in still another manner in the estimation of their molecular weights.[4] Ultrafilters, or membranes of graded permeability, may be made in a number of ways (19), (36), (321), (354). If a membrane is made which is permeable to one protein, but not to another, then the latter may be considered the larger, provided the difference is shown not to depend upon the electrical forces involved. By the use of a series of membranes of varying permeability, Bechhold has arranged the following proteins in the order of the size of their molecules: serum albumin < hemoglobin < gelatin < casein (19). This series has been confirmed, and is now being extended in this laboratory.

Minimal molecular weights estimated from combining weights. The relative estimates of the molecular dimensions of the proteins may be transformed into molecular weights,[4] provided accurate equivalent or containing weights of the proteins are known. Any stoichiometric reaction involving a protein may be utilized in estimating its equivalent weight. From the equivalent combining weight of the reagent the

[4] Ultrafiltration (19) and surface tension (235) measurements reveal the molecular volumes of proteins. The transformation of molecular volumes into molecular weights involves the specific gravities and the water of hydration of proteins. Estimates of the water combined with protein have been made in concentrated salt solutions by Sørensen's "method of proportionality" (322) and in aqueous solutions by Arrhenius' viscosity equation (16). These will be considered in subsequent sections. The exact meaning of the quantities revealed by these two procedures and the errors involved render it undesirable to employ them for these calculations at this time.

amount required to saturate or combine a given weight of the protein can be calculated by means of the relation:

$$\frac{\text{Equivalent combining weight of protein}}{\text{Equivalent weight of reagent}} = \frac{\text{Weight of combined protein}}{\text{Weight of combined reagent}} \quad (1)$$

Certain proteins which are involved in the transport of oxygen in the body, such as the hemoglobins and the hemocyanins, combine chemically with oxygen and with carbon monoxide (148), (273), (287). From the carbon monoxide combining capacity of hemoglobin, Hüfner estimated that its minimal molecular weight was 16,721.

The reactions of proteins with acids or bases may also be employed in the estimation of their equivalent weights. In the accompanying table the equivalent combining weight of egg albumin for acid as determined by electromotive force measurements, and of casein for base as determined by solubility measurements, have been compared with the equivalent weights derived from the composition of these proteins.

Minimal molecular weights estimated from containing weights. The weight containing one atom or molecule of any protein constituent can be calculated from the following relation, provided only its atomic or molecular weight, and the per cent to which it is present in the protein are accurately known.

$$\text{Minimal molecular weight of protein} = \frac{\text{Atomic weight of constituent} \times 100}{\text{Per cent of constituent in protein}} \quad (2)$$

The minimal molecular weight of hemoglobin was estimated in the nineteenth century from the iron it contains (148), (152), (366). Hüfner found that the iron content of the hemoglobin of the ox demanded an equivalent containing weight of 16,619, whereas the carbon monoxide combining capacity had led to an equivalent weight of 16,721. The simultaneous consideration of physicochemical and analytical evidence had therefore led to the minimal molecular weight of a protein as early as 1894 (148).

The copper in the hemocyanins (12), (124), (287), and the sulfur (162), (242), (243), and phosphorus (28) in various proteins give similar information. The weights of egg albumin and of casein that contain one atom of sulfur are recorded in the following table, as is the weight of casein that contains one atom of phosphorus. The excellent agreement between the containing weight of casein calculated on the basis of its phosphorus and sulfur content has long made it certain that the true molecular weight of this protein must be a multiple of 4,300 (28), (98), (168).

Most known proteins are poor in tryptophane and cystine. The unique groups in their molecules, whose presence determines their nutritional significance, also render possible their quantitative determination by colorimetric methods (84). These amino acids are therefore as useful in the calculation of their containing weights as they are essential in animal nutrition.

TABLE 2

The minimal molecular weights of certain proteins

METHOD		AMOUNT OF CONSTITUENT PRESENT	COMBINING OR CONTAINING WEIGHT	ASSUMED NUMBER OF ATOMS OR MOLECULES	MINIMAL MOLECULAR WEIGHT
		per cent	*gram equivalents*	*no. of equivalents*	*gram molecules*
Egg albumin					
Osmotic pressure	(320)				34,000
Tryptophane content	(84)	1.23	16,593	2	33,186
Sulfide sulfur "	(242)	0.491	6,532	5	32,660
Tyrosine "	(84)	4.2	4,312	8	34,496
Sulfur "	(242)	1.616	1,984	17	33,728
Maximal acid-combining capacity	(37)		1,250	27	33,750
Casein					
Tryptophane content	(62) (84)	1.6	12,756	1	12,756
Histidine "	(245)	2.5	6,204	2	12,408
Phosphorus "	(28)	0.71	4,372	3	13,116
Sulfur content "	(98) (168)	0.76	4,220	3	12,660
Equivalent base-combining capacity	(57)		2,100	6	12,600
Ammonia content	(245)	1.61	1,058	12	12,696
Cystine content	(84) (154)	0.25	96,080	2	192,160
Sulfide sulfur "	(242)	0.101	31,752	6	190,512
Minimal molecular weight	(57) (58)		12,800	15	192,000

The simultaneous consideration of the equivalent combining weights and of the elementary and amino acid containing weights of proteins has led to the values of their minimal molecular weights recorded in table 3 (58). The evidence upon which the minimal molecular weights of two of these proteins depends has been arranged in table 2, in order to illustrate the principles involved. Egg albumin was chosen because it is the smallest protein whose molecular weight is well known, and because the osmotic pressure measurements of Sørensen led to a molecular weight that is in excellent agreement with the minimal molecular

weight calculated from Folin and Looney's analyses of its tryptophane and tyrosine contents. The cystine content of casein, which has been reported by two different investigators, and the sulfide sulfur content demand a very high molecular weight of approximately 96,000. Very nearly all the other amino acids, the phosphorus, and the sulfur, have, however, been found in amounts consistent with a minimal molecular weight of 12,800. Twice 96,000 and fifteen times 12,800, or 192,000 becomes the lowest molecular weight that can be ascribed to this substance on the basis of the analytical results that are at present available, and the highest that has thus far been suggested for a protein.

Probable molecular weights of the proteins. The results of the osmotic pressure method and of the ultrafiltration or dialyzing method, taken

TABLE 3

The probable molecular weights of certain proteins

PROTEIN	MINIMAL MOLECULAR WEIGHT*	OSMOTIC PRESSURE MOLECULAR WEIGHT	PROBABLE MOLECULAR WEIGHT
Egg albumin	33,800	34,000 (320)	33,800
Serum albumin	5,000	45,000 (**)	45,000
Hemoglobin	16,666	48,000 (290)	50,000
Edestin	29,000		58,000
Hemocyanin, *Limulus*	22,700		68,100
Pseudoglobulin	27,000	80,000 (**)	81,000
Gelatin	10,300	96,000 (315)	
Euglobuin		140,000 (**)	135,000
Casein	12,800		192,000

* The data upon which these minimal molecular weights depend have previously been considered (58).

** Unpublished data of S. P. L. Sørenson.

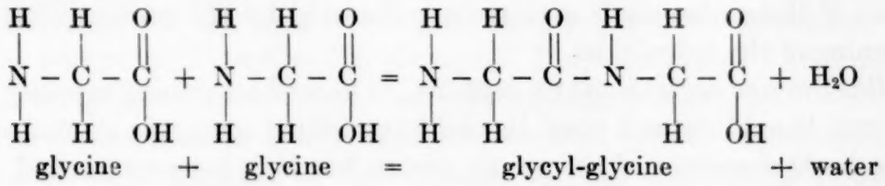
in conjunction with the minimal molecular weights of the proteins enable the estimation of their probable molecular weights. Certain proteins have been arranged in table 3 in what appears at the present time to be their relative molecular volumes as determined by ultrafiltration and dialyzing experiments. Their minimal molecular weights have been reported in the first column and the estimates of their molecular weights based upon osmotic pressure measurements, in the second. In the last column their probable molecular weights have been estimated as integral multiples of their minimal molecular weights.[5] Some doubt

[5] These estimates of the probable molecular weights are in certain cases somewhat smaller multiples of the minimal molecular weights than previously reported (53), and have been suggested by further dialyzing experiments.

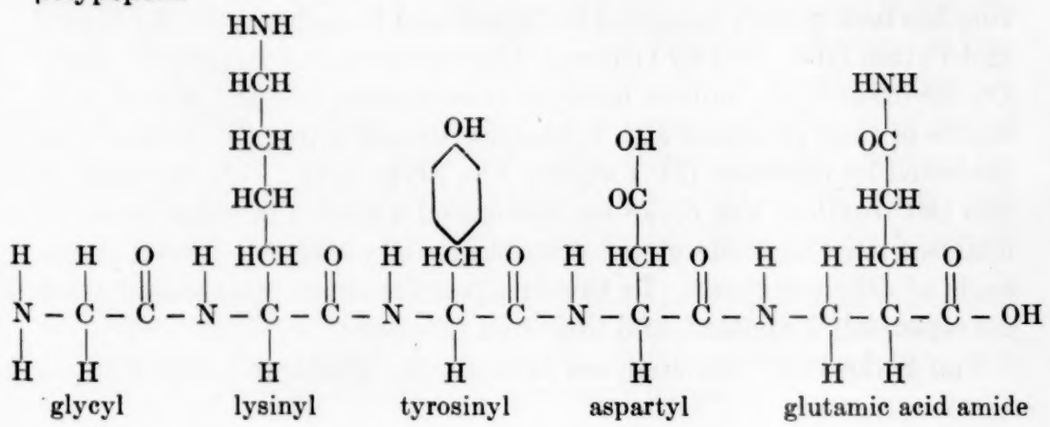
remains as to the number of times the minimal molecular weights of certain of the proteins must be multiplied in order to yield their true molecular weights.

Comparison of the molecular weights of egg albumin, serum albumin and serum globulin determined in this way, with the estimates derived by Sørensen and his collaborators from their osmotic pressure measurements, leaves little doubt that the two procedures are capable of giving similar results. It therefore seems probable that the molecular weights, at least of certain proteins, are now well known.

III. THE RELATION BETWEEN THE BASIC AMINO ACIDS IN PROTEINS AND THEIR ACID COMBINING CAPACITY. The structure of the protein molecule, and of the amino acids of which proteins are composed, must determine both the nature and the number of the reactive groups which proteins possess. Fischer (83) demonstrated that amino acids combined with each other to form polypeptids by condensation of the amino group of one acid and the carboxyl group of another, with the loss of one molecule of water.



When several monoamino monocarboxylic acids are combined with each other in this manner, they yield no free groups other than those at the very ends of the chain. The structure may become more complicated, however, if dibasic or dicarboxylic acids are present. For basic amino acids, when held in polypeptid linkage, still contain one free basic group; and dicarboxylic acids, one free carboxyl group. The type of structure that results is represented by the following hypothetical polypeptid.



These groups may remain free in polypeptids and in the protein molecule, or may give rise to a large number of chains. Which group of the amino acids are combined to form the protein molecule and which remain free is of no consequence in determining the number of free groups. The number of possible free acid groups must be equal to the difference between one more than the total number of acid groups and the number of amino acids in the molecule.

$$\text{Free acid groups} = \text{Total acid groups} + 1 - \text{amino acids} \qquad (3)$$

A comparable relation exists between the number of amino acids and the number of basic groups. The actual number of active groups can, of course, be reduced by the combination of acid groups with basic groups to form cyclic anhydrides.

Certain recent investigators believe that cyclic anhydrides consisting of condensed monoamino monocarboxylic acids may be held in proteins by forces other than those of primary valence (125), (126), (170). The groups of such acids if they are present would complicate our calculations if the amino acids continued to be held by the protein after the opening of the anhydrides.

Basic amino acids in certain proteins. The acid combining capacity of a protein should depend upon the acid combining groups in its molecule. The basic character of the amino groups has long been recognized, and an increase in their number ought, therefore, to increase the acid combining capacity of the protein. In recent years technical advances have been made in the estimation of the diamino acids in proteins, and of their acid combining capacity, which make it possible to test this relation.

Advances of two kinds have increased our knowledge of the diamino acid contents of various proteins. On the one hand the separation of diamino acids from protein hydrolysates and their quantitative estimation has been greatly improved by Kossel and Kutscher (159), by Kossel and Patten (160), and by Osborne, Leavenworth, and Brautlecht (250). On the other hand, indirect methods of estimating free amino groups by means of their reactions with formaldehyde and nitrous acid have been perfected by Sørensen (316) and by Van Slyke (342). On the basis of this last reaction, Van Slyke has elaborated a system in terms of which analyses may be made of the distribution of nitrogen between amino acids of different types. In this analysis the three basic amino acids are separately estimated, and their sum revealed.

Van Slyke (342) has analyzed hemoglobin, gliadin, edestin, gelatin,

fibrin, and *Limulus* hemocyanin by his method; Hartley (109) has employed it in the study of serum albumin and various fractions of serum globulin; and Lüscher (200) of Bence-Jones' protein. Attempts to differentiate between preparations derived from different sources led Cross and Swain (61) to study the nitrogen distribution of a large number of samples of gliadin and glutenin, and a similar problem led Plimmer and Shimamura (282) to study various gelatin preparations. The results of these investigations have been recalculated from nitrogen, as per cent of nitrogen, to mols of amino acid in one gram of protein,[6] and arranged in table 6. There have been added to this table the determinations of Osborne, Jones, and Leavenworth (248) of the diamino acids in egg albumin, of Osborne and Guest (245) in casein, of Kossel and Kutscher (159), and of Osborne and Liddle (252) in zein.

Acid combining capacity of certain proteins. In 1898 Bugarszky and Liebermann (37) demonstrated, by means of electromotive force measurements, that egg albumin combined with both acids and bases, and in 1899 Osborne (239) demonstrated by a comparative colorimetric method that various amounts of acid were required to neutralize the basic groups of a number of proteins. Bugarszky and Liebermann studied the electromotive force between hydrogen electrodes immersed in a cell composed of 0.05 N hydrochloric acid, and in one containing the same concentration of the acid and varying amounts of albumin. From the initial concentration of the acid and the electromotive force measurements, the concentrations of free acid were calculated by means of the relation:

$$\text{Electromotive force} = \frac{RT}{nF} ln \frac{C_2}{C_1} \qquad (4)$$

in which R is the gas constant, T the absolute temperature, nF the electrochemical equivalent, C_1 the concentration of hydrogen ions in the standard cell, and C_2 the concentration of hydrogen ions in the cell containing protein (46), (233), (299). The difference between the free acid and the total acid present yields the acid combined with the protein. A recalculation of Bugarszky and Liebermann's results is tabulated below, in part because of historical significance, and in part because the results are as accurate as measurements that have subsequently been made on egg albumin solutions. These measurements demonstrate

[6] In the calculations, 17.66 has been taken as the per cent of nitrogen in gliadin (240), 18.69 in edestin (249), 17.97 in gelatin (202), 16.91 in fibrin (202), 17.70 in hemoglobin (202), 16.18 in hemocyanin (12), 15.93 in serum albumin (202), 15.85 in serum globulin (202), and 16.21 in Bence-Jones' protein (141).

that in the presence of an appreciable concentration of acid, the albumin was saturated with the acid. A more than tenfold increase in free acid resulted in a very small increase in combined acid. These calculations thus reveal the maximum acid combining capacity of the protein.

Since the researches of Bugarszky and Liebermann, the electromotive force method has been employed in a large number of investigations of the acid and base combining capacities of different proteins. Although electromotive force measurements upon systems containing proteins and acids, or proteins and bases, can be made with great accuracy, the calculation from such measurements of the acid, or base, combining capacity of the protein is not free from error.

Electromotive force measurements do not yield the hydrogen ion concentration. They yield the hydrogen potential (46), (317), which is

TABLE 4

Acid combining capacity of egg albumin calculated from the results of Bugarszky and Liebermann (37)

ALBUMIN IN SYSTEM	HCl IN SYSTEM	HCl FREE	HCl COMBINED	HCl COMBINED BY 10 GRAMS ALBUMIN
p	a	b	$a-b$	$(a-b)/p$
grams	N	N	N	N
6.4	0.05	0.0027	0.0473	0.0074
3.2	0.05	0.0262	0.0238	0.0074
1.6	0.05	0.0373	0.0127	0.0079
0.8	0.05	0.0436	0.0064	0.0080
0.4	0.05	0.0468	0.0032	0.0080

the negative logarithm of the hydrogen ion activity. The hydrogen ion activity may be considered the product of the stoichiometric concentration of hydrogen ions, and the activity coefficient, γ;

$$\text{hydrogen ion activity} = \gamma\ (\text{hydrogen ion concentration}) \tag{5}$$

or:

$$\text{pH}^{+} = \log \frac{1}{\gamma\ (\text{hydrogen ion concentration})} \tag{6}$$

Only when the activity coefficient, γ, is equal to one does the hydrogen potential, pH, measure the hydrogen ion concentration. The activity coefficient of the hydrogen ion varies greatly, however, and is not only influenced by the concentration of hydrogen ions, but also by the concentration, the valence, and the dimensions of all the ions in the solution, and by the dielectric constant of the solution (68), (69), (103), (176).

In systems containing hydrochloric acid and a protein hydrogen potential measurements indicate combination between the protein and the acid. On the other hand the chloride potential measurements of Pauli and his collaborators (201), (238), (263), (270), and of Hitchcock "indicate that the chlorides" of the proteins that have thus far been carefully studied "are highly ionized electrolytes, ionizing to yield chloride ion and a positive protein-hydrogen ion" (130, p. 394). Some combination with chloride ions appears to occur with certain proteins, but in dilute solutions such combination is lower than that with hydrogen ions. Chloride ions as well as hydrogen ions affect the activity coefficient of the hydrogen ion. The protein ions themselves must theoretically affect the activity coefficient, and the behavior of certain proteins in very acid solutions suggests that they may. Uncertainty regarding the valence of proteins has made it impossible, however, to take into account the influence of the protein ion. Up to the present, the most accurate estimates of the acid and base combining capacities of proteins have been made by correcting only for the other ions present. This was accomplished in the researches of Bugarszky and Liebermann and of Robertson by immersing the reference hydrogen electrode in a solution of the same total acid concentration as the protein solution. The very low concentration of acid and of protein, in most of the other researches that have been considered, renders it probable that the calculations that have been made are substantially correct, though they are still subject to correction for the effect of the protein ion.[7,8]

The error in estimating combined acid becomes greater, the greater the concentration of total acid, for two reasons. In the first place, the activity coefficient is more variable in concentrated than in dilute

[7] Certain investigators have calculated their results as though the activity coefficient of the hydrogen ion were determined not by the total ionic concentration, but only by the free acid revealed by hydrogen potential measurements. Such calculations involve the assumption that proteins combine with the chloride ion to the same extent as they combine with the hydrogen ion. This, as we have seen, is not borne out by recent investigations (130), (263). Such measurements have accordingly been recalculated by the method that has elsewhere been described in detail (54). We are indebted to D. I. Hitchcock for furnishing us with his electromotive force measurements for recalculation. W. A. Hoffman and R. A. Gortner prefer the method of calculation employed by them (136) to that adopted in this review. Reference may be made to their paper for their reasons for this, and also for the different conclusions to which they have come.

[8] I am indebted to R. E. L. Berggren for recalculating and reinvestigating the combination of proteins with acids and bases in such a way as to unify and supplement existing data.

solution; in the second place, since the amount of acid combined with a protein is determined by the difference between the total acid and the free acid, the error in calculating acid combining capacity is least in systems containing relatively small amounts of free acid. Where the amount of free acid becomes three or four times as great as the amount of acid bound by the protein, this error becomes formidable. Lloyd and Mayes (185), Izaguirre (151), and Hoffman and Gortner (136) have attempted to draw conclusions regarding acid and base combining capacities in such systems. The latter have concluded that, although the reactions of proteins are chemical between pH 2.5 and 10.5, "physical adsorption appears to be the predominating if not the sole factor" in the acid and alkaline regions beyond these limits where "all proteins tested regardless of their chemical combination or biological source bound essentially the same amount of acid or alkali per gram of protein at any given pH" (136, p. 356). Certain of the measurements which led Hoffman and Gortner to draw this conclusion have been recalculated over the range in which the amount of free acid increased from less than one-half to more than eight times that bound. These measurements represent all those reported upon casein at temperatures between 20 and 25°C., and at concentrations of hydrochloric acid lower than 0.1 N. The activity coefficients of Lewis and Randall (176) have been employed in the recalculation. So calculated, these measurements are not very different from those reported by different investigators, excepting in so far as Hoffman and Gortner's results appear to show a variation of combining capacity with temperature. Their measurements at 15 and at 35°C. show larger variations than those reported in Table 5.

It seems more reasonable to conclude from measurements made in strongly acid and alkaline solutions that the irregularities in acid or base combining capacity result from incipient hydrolysis or from the effect of proteins upon the activity coefficients of the hydrogen and hydroxyl ions, than to conclude that such measurements demonstrate either further combination or adsorption. Studies of the effect of proteins upon the activity coefficients of the hydrogen and hydroxyl ions, comparable to those of Arrhenius (14), of Harned (103), and of others (33), (220), (327), (338), (358) upon the effects of neutral salts upon the activity coefficients of these ions, are needed before we can understand the effects of weak electrolytes upon strong electrolytes, and of strong electrolytes upon weak electrolytes.

The acid combining capacity of serum albumin was studied by Manabe and Matula in 1913 (201), and by Pauli and Hirschfeld in

1914 (267). Their results have been recalculated by means of the more accurate activity coefficients that are at present available (176), and yield the result that serum albumin can combine 153 and 156 $\times 10^{-5}$

TABLE 5

Acid combining capacity of one per cent casein calculated from the results of Hoffman and Gortner (136)

TABLE	T	pH*	HCl IN SYSTEM	HCl FREE	HCl COMBINED BY 10 GRAMS CASEIN
			a	b	$a-b$
			N	N	N
xxxiv	**	2.473	0.0120	0.0037	0.0083
lxxx	22°	2.392	0.0123	0.0044	0.0079
lxxx	22°	2.249	0.0148	0.0062	0.0086
lx	25°	2.299	0.0120	0.0055	0.0065
lxxx	22°	2.155	0.0172	0.0077	0.0095
lxxx	22°	2.096	0.0196	0.0089	0.0107
xxxiv	**	2.062	0.0180	0.0096	0.0084
lx	25°	1.980	0.0180	0.0116	0.0064
lxxx	22°	1.978	0.0244	0.0117	0.0127
xxxiv	**	1.845	0.0240	0.0160	0.0080
lxxx	22°	1.843	0.0291	0.0161	0.0130
lx	25°	1.802	0.0240	0.0176	0.0064
xxxiv	**	1.775	0.0300	0.0188	0.0112
lx	25°	1.698	0.0300	0.0225	0.0075
xxxiv	**	1.555	0.0450	0.0316	0.0134
lx	25°	1.488	0.0450	0.0369	0.0081
xxxiv	**	1.372	0.0600	0.0488	0.0112
lx	25°	1.362	0.0600	0.0499	0.0101
xxxiv	**	1.258	0.0750	0.0643	0.0107
lx	25°	1.251	0.0750	0.0653	0.0097
xxxiv	**	1.174	0.0900	0.0791	0 0109
lx	25°	1.160	0.0900	0.0817	0 0083

* The hydrogen potentials given by Hoffman and Gortner have been retained in this recalculation. If their electromotive force measurements are recalculated by means of Lewis, Brighton and Sebastian's (174) value for the calomel electrode, slightly higher pH values are obtained. Hoffman and Gortner have used another method in calculating their results.[7]

** "The experiments were carried out at room temperature, 20–22°C." (136, p. 271).

mols of hydrochloric acid per gram. Two serum albumin preparations—one of them crystalline—that have been studied in this laboratory yield 160 and 150 $\times 10^{-5}$ for the maximum acid combining capacity of this

protein. These results are consistent with each other, and reveal an acid combining capacity only slightly greater than that demanded by Hartley's estimate (109) of the diamino acids in serum albumin.

The diamino acids in gelatin are better known than those in serum albumin (282), (342). Blasel and Matula (25) studied the acid combining capacity of this protein in 1913. Their results are very much higher than those of Loeb (195) and Hitchcock (128), (130), which calculated yield 92×10^{-5}, 86×10^{-5}, and 89×10^{-5} mols of acid per gram protein, respectively, as the maximum acid combining capacity of this protein. In this case, the protein would seem to contain more diamino acids than are required to account for its acid combining capacity.[9]

Blasel and Matula (25) also studied a gelatin preparation that they believed had been deaminized by treatment with formaldehyde, and found only a slightly lower acid combining capacity than for ordinary gelatin. The interpretation that has often been placed upon their experiment is that the acid combining capacity of protein could not be related to the free amino groups, since deaminized gelatin could still combine acid, and that it must, therefore, depend upon the—COHN—groups in the protein molecule (299), (300). Hitchcock (131) again studied the acid combining capacity of deaminized gelatin, and found that treatment with nitrous acid decreased the acid combining capacity from 89 to 44×10^{-5} mols of hydrochloric acid per gram gelatin, an amount very nearly equal to the lysine in the gelatin molecule. Since the gelatin molecule contained enough diamino acids to account for its acid combining capacity, and since deaminized gelatin had a lower combining capacity, it is probable that the free rather than the internal groups in the protein molecule are those that are involved in combining acids.

Hitchcock has also studied the acid combining capacity of edestin and serum globulin. In both cases the concentration of the basic amino acids has been found slightly greater than the acid combining capacity. These and our own measurements upon serum globulin, casein, Bence-Jones' protein, gliadin and zein render it certain that there are enough of these groups in the protein molecule to account for its acid combining capacity.

[9] If the histidine of Dakin (64) be taken, rather than that of Van Slyke on the basis that his previous figure included a new monoamino acid (343) the agreement between diamino acids and acid combining capacity is still better. Dakin found but 0.9 per cent histidine, or 5.8×10^{-5} mols per gram. The total diamino acids thus become 93.5×10^{-5} mols per gram, while the best value for acid combining capacity is 89×10^{-5} mols per gram.

TABLE 6

The relation between the basic amino acids in proteins and their acid combining capacity

PROTEIN	BASIC AMINO ACIDS IN PROTEIN	BASIC AMINO ACIDS IN 1 GRAM PROTEIN				ACID COMBINING CAPACITY OF 1 GRAM PROTEIN
		Histidine	Arginine	Lysine	Total	
	per cent	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$
Edestin..... (342)	23.4	25.3	90.3	25.7	141.3	127
Hemocyanin, *Limulus*.. (342)	22.9	50.4	45.5	49.0	144.9	
Hemoglobin..... (342)	22.4	52.9	24.3	68.8	146.0	
Serum albumin..... (109)	21.5	21.9	28.2	90.3	140.4	155
Fibrin..... (342)	20.4	19.2	41.9	69.4	130.5	
Gelatin..... (342)	17.1	18.9	47.2	40.5	106.6	89
Pseudoglobulin, ox..... (109)	16.0	17.9	30.6	54.3	102.8	85
Euglobulin, ox..... (109)	15.5	14.2	32.8	52.0	99.0	
Casein..... (57)	14.7	16.1	21.9	57.3	95.3	90
Bence-Jones..... (200)	14.1	17.3	26.8	46.5	90.5	92
Egg albumin..... (248)	10.4	11.0	28.2	25.7	64.9	80
Gliadin..... (342)	7.2	21.6	18.0	4.7	44.3	34
Zein..... (65)	2.6	5.3	10.5	0.0	15.8	0

Edestin: Osborne (239) found that 127 $\times 10^{-5}$ mols of hydrochloric acid were required to titrate 1 gram edestin to tropaeolin. Hitchcock (127) (130), has studied edestin twice. A recalculation of his results yields 126 and 124, and a recalculation of Kodama's (157), from 109 to 118 $\times 10^{-5}$ mols as the maximum acid combining capacity. The acidity of Kodama's preparations and of the preparations employed by Hitchcock suggests that they may have been edestin hydrochloride. In that case their acid combining capacities should be increased by 7 $\times 10^{-5}$ according to Osborne (241). Therefore 127 $\times 10^{-5}$ mols per gram may still be taken as the maximum acid combining capacity of this protein.

Serum albumin: A recalculation of Manabe and Matula's (201) and Pauli and Hirschfeld's (267) results by means of newer (176) activity coefficients for the hydrogen ion brings the maximum acid combining capacities to 153 and 156 $\times 10^{-5}$ mols per gram. Two preparations studied in this laboratory bound 150 and 160 $\times 10^{-5}$. Therefore 155 $\times 10^{-5}$ mols per gram may be taken as the maximum acid combining capacity.

Fibrin: Bracewell believed that 96 $\times 10^{-5}$ mols per gram was the maximum acid combining capacity of fibrin (30). Hoffman and Gortner have studied fibrin in different experiments (136), but although these point to a higher acid combining capacity than Bracewell suggested, they are not sufficiently consistent with each other to permit any quantitative estimate to be made. See also the results of Trendtel (339).

Gelatin: Although Blasel and Matula's (25) results are much higher, three investigations, one by Loeb (195, p. 50, first ed.) and two by Hitchcock (128) (130), suggest 89 $\times 10^{-5}$ as the maximum acid combining capacity of this protein.

Their results recalculated yield 92, 89 and 86 $\times$ 10^{-5} mols per gram. See also (17), (108), (185).

Serum globulin: Two electromotive force measurements of Robertson upon serum globulin at pH 2.43 and 2.14 lead to 77 and 83 $\times$ 10^{-5} mols per gram as the acid combining capacity. Comparable results have been obtained by Hitchcock (129) (130). His electromotive force measurements recalculated indicate a maximum at 85 $\times$ 10^{-5}. This preparation had a pH of 6.17, however, and at least 4 $\times$ 10^{-5} mols must have been required to neutralize the base in the preparation. Thus 81 $\times$ 10^{-5} becomes the probable acid combining capacity of this preparation. Another preparation studied by Hitchcock had a much lower acid combining capacity of 72 $\times$ 10^{-5} (129), although its reaction was isoelectric. Whether in this case he was studying a different globulin fraction, or whether he was studying a hydrochloride containing acid cannot be decided at this time. His other preparation agrees with Robertson's results in suggesting that the maximum acid combining capacity of serum globulin is in the neighborhood of 85 $\times$ 10^{-5} mols per gram.

Casein: Bracewell (30) estimated that casein combined 60 $\times$ 10^{-5} mols acid per gram. Hitchcock's (130) and Loeb's (190) results, recalculated, show 59 and 72 $\times$ 10^{-5} mols per gram as the maximum acid combining capacity of casein. Measurements made by R. E. L. Berggren in this laboratory upon preparations whose base combining capacities have already been reported (54) show that acid combining capacities also increase when the casein is modified by alkalis. Three states have been noted, corresponding to the three base combining states and combining approximately 60, 76, and 90 $\times$ 10^{-5} mols per gram respectively. Hitchcock's and Loeb's results fall in the first and second class, and Hoffman and Gortner's results (136) at hydrochloric acid concentrations lower than 0.1 N, recalculated by means of the activity coefficients of Lewis, into the second and third groups. At more acid reactions, Hoffman and Gortner's results run higher, but the experimental error is greater there.

Bence-Jones' protein: Two Bence-Jones' protein preparations have been studied in this laboratory. The one, crystallized at pH 5.75 and combined 92 $\times$ 10^{-5} mols per gram. The other preparation had a pH of 6.12 and combined 109 $\times$ 10^{-5}. From this latter result 4 $\times$ 10^{-5} must be subtracted in order to compare it with the former, since that amount of acid was required to bring it to the pH of the crystalline preparation. It is of course possible that the crystalline preparation was not isoelectric, but probably yielded the most accurate results that are available.

Egg albumin: Bugarszky and Liebermann's (37) classic researches yield 80 $\times$ 10^{-5} mols per gram as the maximum acid combining capacity of egg albumin. Osborne found that 90 $\times$ 10^{-5} mols were required to titrate crystallized egg albumin to tropaeolin (239). Hitchcock in a more recent investigation found a combining capacity of 92 $\times$ 10^{-5}, but his preparation had a pH of 5.67 (130). His data, recalculated and corrected for the amount of acid (15 $\times$ 10^{-5}) required to bring 1 gram to the isoelectric point, average 77, and render 80 $\times$ 10^{-5} mols per gram a most probable value for the combining capacity.

Gliadin: Bracewell gives a maximum acid combining capacity of gliadin as 28 $\times$ 10^{-5} mols (30). Hoffman and Gortner's (136) measurements rise with increasing acidity, but vary only from 29 to 35 $\times$ 10^{-5} from pH 2.5 to pH 2.1, over

which range the concentration of free acid becomes more than double that bound. Two preparations studied by R. E. L. Berggren in this laboratory had a combining capacity of approximately 34×10^{-5}, until the amount of free acid was five times that bound. Thereafter the combining capacity apparently increased as did the turbidity of the solutions. Lüer's (198) results are not included since they do not in the least agree with those considered.

Zein: Although Hoffman and Gortner (136) report an acid combining capacity for zein, Cohn, Berggren, and Hendry (55) were unable to discover any basic properties. In their experiments zein neither dissolved in acid nor did it combine with acid.

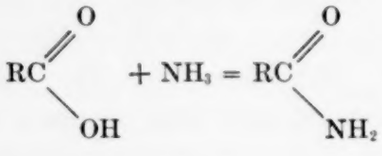
The excess of basic groups over acid combining capacity led Bracewell to suggest that, when bound in polypeptid linkage, lysine and arginine, but not histidine, were capable of combining with acids (30). His estimates of acid combining capacity depended upon an indirect method, and have generally been lower than those derived from electromotive force measurements. The most accurate values that have now been obtained, unquestionably remain lower than the expectation from all the basic amino acids present, but are higher than the sum of the arginine and lysine alone. Moreover there does not seem to be any theoretical reason for believing that histidine may not be capable of combining acid.

A more extraordinary condition obtains in zein. This protein contains both histidine and arginine in small but unmistakable amounts (65). Hoffman and Gortner report hydrogen potential measurements upon systems containing zein and hydrochloric acid which appear to show combination with acid. On the other hand experiments conducted in this laboratory revealed no apparent acid combining capacity; zein neither combined with acids, nor dissolved in acids (55). With respect to its physicochemical behavior, it appeared, therefore, not to be an amphoteric substance.

The results that are at present available indicate that on the whole proteins rich in basic amino acids have high acid combining capacities, while those that are poor in this respect have low acid combining capacities. In the case of egg albumin the basic amino acids have not been estimated by the nitrous acid method, but by isolation. The estimates of the basic amino acids in Bence-Jones' protein by Hopkins and Savory (141) are also lower than the acid combining capacity of this protein. It is desirable that the basic amino acids of these proteins and also of serum albumin be reanalyzed. With these exceptions the proteins thus far investigated contain a sufficient concentration of basic amino acids to account for their acid combining capacities.

It is noteworthy that the three proteins that contain the highest concentration of basic amino acids are all relatively neutral proteins. The basic character of the edestin molecule has been studied by Osborne (241), and the high concentration of basic amino acids in the hemoglobins and in *Limulus* hemocyanin suffices not only to neutralize the acidity of the prosthetic groups in these molecules but also to render them neutral proteins.

IV. THE RELATION BETWEEN THE DICARBOXYLIC ACIDS IN PROTEINS AND THEIR BASE COMBINING CAPACITIES. It was previously believed that there were not enough free carboxyl groups in protein molecules to account for their base combining capacities. This depended upon the knowledge that most proteins, upon acid hydrolysis, yielded a certain percentage of their nitrogen as ammonia. This nitrogen represents, as was early recognized, amide groups which have combined with free carboxyl groups in a manner that may be represented by the relation:



(7)

Early analyses (250) appeared to indicate that there was approximately enough amide nitrogen in many proteins to neutralize the carboxyl groups derived from dicarboxylic acids, and that those proteins that contained the largest number of dicarboxylic acids, also contained the largest amount of ammonia.

Dicarboxylic acids in certain proteins. Whereas the methods of determining the ammonia that were employed in these early investigations were very accurate, the methods of isolating dicarboxylic acids were far from quantitative. As we have seen, β-hydroxyglutamic acid was discovered only in 1918. Moreover Dakin has been able to increase the yields of dicarboxylic amino acids in casein from 16.9 to 36.4 per cent (62), and in zein from 27.9 to 35.6 per cent (65). These results have been obtained by means of his butyl alcohol extraction method of removing the monoamino monocarboxylic acids, and by applying Foreman's method for the separation of the dicarboxylic acids from protein hydrolysates (85). These new estimates of the dicarboxylic acids are in excess of the amide nitrogen and indicate that these proteins contain free carboxyl groups.

Dakin has thus far studied only casein (62), gelatin (64), and zein (65) by the new methods. The dicarboxylic acids in these proteins

TABLE 7

The relation between the dicarboxylic acids in proteins and their base combining capacity*

PROTEIN	DICARBOXYLIC AMINO ACIDS IN 1 GRAM PROTEIN					BASE COMBINING CAPACITY OF 1 GRAM PROTEIN
	Glutamic acid	Aspartic acid	β-hydroxy-glutamic acid	Amide bound acids	Free dicarboxylic acids	
	a	*b*	*c*	*d*	$a+b+c-d$	
	mols $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$	*mols* $\times 10^{-5}$
Casein, Hammarsten..	148.0 (85)	30.8 (62)	64.4 (62)	94.5(245)	148.7	183
Casein, unmodified....						138
Egg albumin.....						80
Gelatin.....	39.4 (64)	25.5 (64)	0.0 (64)	23.5 (64)	41.4	57
Gliadin.....			14.7 (63)			30
Zein.....	212.8 (65)	13.5 (65)	15.3 (65)	211.5(252)	30.1	30

* The mols of tyrosine per gram for these proteins, are casein, 29.6; egg albumin, 23.2; gelatin, 0.0; gliadin, 19.3; and zein 30.9 $\times 10^{-5}$ (84).
The amounts of the other hydroxy acids are not accurately known.

Casein: Robertson (296) gives 180 $\times 10^{-5}$ mols per gram as the maximum base combining capacity of casein "nach Hammarsten". Cohn and Berggren (54) obtained an almost identical value for casein prepared in a similar manner, but they and Greenberg and Schmidt (93) have found that casein that has not been prepared in this way has a lower base combining capacity of 155 and 160 respectively. In two preparations Cohn and Berggren found even lower base combining capacities under conditions such that cyclic anhydrides may not have been hydrolyzed, of 136 and 140 $\times 10^{-5}$ mols per gram.

Egg albumin: Bugarszky and Liebermann (37) obtained a maximum base combining capacity of 71 $\times 10^{-5}$ mols per gram. Measurements made in this laboratory by R. E. L. Berggren indicate a slightly higher result of approximately 80 $\times 10^{-5}$ mols per gram.

Edestin: The largest base combining capacity of edestin noted by Kodama (157) was 75 $\times 10^{-5}$ mols. Three measurements of Hitchcock (127) upon recalculation suggest a higher base combining capacity, probably in the neighborhood of 90 $\times 10^{-5}$ mols per gram.

Gelatin: The maximum base combining capacity of gelatin is extremely well known. A recalculation of Loeb's (188) and Hitchcock's (133) electromotive force measurements yield 56, 57, and 56 $\times 10^{-5}$ mols per gram as the combining capacity. Greenberg and Schmidt (90) report an almost identical value of 60 $\times 10^{-5}$ mols per gram, and this is the highest base combining capacity that Atkin and Douglas' (17) measurements reveal upon recalculation.

Gliadin: Greenberg and Schmidt (90) report a base combining capacity of 30 $\times 10^{-5}$ mols per gram. This value has been comfirmed by measurements made in this laboratory. Hoffman and Gortner's (136) measurements are of

this order of magnitude until more than six times as much base is free as combined.

Zein: Two zein preparations bound 28 and 31 $\times 10^{-5}$ mols sodium hydroxide per gram (55). Hoffman and Gortner's (136) measurements reveal no maximum base combining capacity.

expressed as mols of amino acid per gram protein are recorded in table 7, and their sum diminished by the mols of amide nitrogen in the molecule. Dakin has also estimated the amount of β-hydroxyglutamic acid in gliadin and glutenin. Osborne believes that there is no β-hydroxyglutamic acid in edestin. The old values for the dicarboxylic acids in these proteins are all too low, however, and are therefore not considered.

Base combining capacity of certain proteins. The maximum base combining capacities of a number of proteins have now been adequately investigated. Robertson (296) found that 1 gram of casein "nach Hammarsten" could combine 180 $\times 10^{-5}$ mols of sodium hydroxide per gram, and Cohn and Berggren (54) have found an almost identical base combining capacity of 183 $\times 10^{-5}$ mols for casein similarly prepared. Casein that has not been modified in the course of its preparation has a lower base combining capacity, of approximately 138 $\times 10^{-5}$ mols per gram (54). This figure is lower than the concentration of the free dicarboxylic acids in casein. The free dicarboxylic acids alone are not sufficient to account for the higher base combining capacity of casein that has been modified by alkali. It has been suggested by Harris (107) that tyrosine should be considered a dibasic acid, and Greenberg and Schmidt (93) have assumed that tyrosine might in this case be functioning as an acid. If the phosphorus in the casein molecule be considered phosphate phosphorus, and therefore possessed of acid properties, another method of accounting for the base combining capacity is presented. The change in base combining capacity produced by alkali has been ascribed by Greenberg and Schmidt to the splitting off of amide groups, and by Cohn and Berggren to the opening of internal anhydrides. These changes are probably accompanied by racemization of the casein (66).

The caseins that have been investigated thus far fall into three groups, having base combining capacities of approximately 138, 155, and 183 $\times 10^{-5}$ mols per gram. Hammarsten casein falls in the last group, while casein that has been brought to a hydrogen ion concentration between 10^{-7} and 10^{-8} N in the course of its preparation shows the intermediate capacity, and casein that has never been brought to an alkaline reaction, the lowest. The difference between the last two is almost

exactly equal to the difference between the acid combining capacities of different casein preparations (table 6) and therefore presumably represents cyclic anhydrides. These apparently hydrolyze in casein in most faintly alkaline solutions, increasing both the acid and base combining capacities. The increase in base combining capacity may also, in part, represent the splitting off of amide groups, since the increase in acid combining capacity is somewhat lower.

That the denaturation of proteins may occur at very slightly alkaline reactions has also been suggested by Pauli (263) and recently by Wu (365). Wu has demonstrated that serum globulin, that has been brought to faintly alkaline reactions in the course of its preparation, behaves in a quite different manner than globulin that has not been so treated. Comparable studies upon other proteins besides casein and serum globulin may reveal the presence of specific groups in the molecule and explain the more irregular results that have often been reported when proteins were studied in alkaline, rather than in acid, solution[10] (263).

Gelatin was titrated with sodium hydroxide to very alkaline reactions by Hitchcock (133). A recalculation of his results yields 56×10^{-5} mols per gram as the maximum base combining capacity of this protein. Greenberg and Schmidt (90) report a slightly higher value, 60×10^{-5} mols per gram. The agreement is excellent. In gelatin, as in casein, the base combining capacity is slightly higher than the free dicarboxylic acids, but smaller than these plus tyrosine. The relation between free acid groups and base combining capacity is clearly significant, despite the discrepancies that have been pointed out. Thus there are nearly four times as many free dicarboxylic acids in casein as in gelatin, and the former protein possesses three times the base combining capacity of the latter.

Zein, the only other protein thus far investigated by Dakin, has precisely the same base combining capacity as may be calculated from the free dicarboxylic acids that it contains (55). On the other hand it possesses, as we have seen, diamino acids that exert no acid combining capacity, and may therefore be internally bound. It is possible that further refinements in technique will still further increase the yields of dicarboxylic acids in this, as in other proteins. If tyrosine be considered a dibasic acid, however, there are enough reactive groups to account for both the acid and basic behavior of zein, as of gelatin and casein.

[10] This is also indicated by the more irregular results in the alkaline than in the acid titration curves of certain proteins.

Gliadin has not been reinvestigated by means of the newest methods of separating dicarboxylic acids. If Dakin's estimate of β-hydroxyglutamic acid and Folin and Looney's estimate of tyrosine are added to older analyses of aspartic and glutamic acids, the number of free acid groups comes very close to the base combining capacity (90). On the other hand, a yield of aspartic acid far greater than that previously recorded has been obtained from gliadin by Osborne and Vickery.[11] The base combining capacity of this protein, at the reactions investigated, may possibly be accounted for on the basis of free dicarboxylic acids without recourse to hydroxyacids.

The base combining capacities of a number of other proteins have been studied and are reported. It is noteworthy that the prolamins possess the smallest base combining capacities, as well as the smallest acid combining capacities. These proteins must therefore be considered, on the basis of their weight, as very poor electrolytes. Next in sequence comes gelatin, very much poorer in acid than in basic groups, and from this point of view a very abnormal and electrically unbalanced protein. At the other end of the series come the more soluble proteins. Of these only one, lactalbumin, has been adequately analyzed (155), but its base combining capacity has unfortunately not yet been studied.

The analytical and physicochemical information, that is being considered, has one serious defect. It is derived from different sources. Were it possible to compare the diamino and dicarboxylic acids of a protein preparation with its acid and base combining capacity, the evidence would be more conclusive than that collected from diverse sources.

V. The number of acid and of base combining groups in proteins. The number of acid and of base combining groups in a molecule can be calculated from the capacity of a protein to combine with acids and bases, provided its molecular weight is known. In the previous discussion, the combining capacities of proteins have been recorded as mols of reagent per gram protein. The reciprocals of these values yield the equivalent weights of the proteins. These have been calculated from the experimental results that have been considered. The molecular weight of a protein, divided by its equivalent weight yields the number of acid or of basic groups in the molecule. In those cases in which the true molecular weight of the protein is not yet established, its minimal molecular weight, multiplied by a coefficient n has

[11] Personal communication.

been substituted. This coefficient represents the ratio of the true molecular weight to the minimal molecular weight, and therefore also of the number of reacting groups in the molecule to the number of groups in its integral fraction, the minimal molecular weight.

The albumins that have been studied thus far have both small molecular weights and a large number of dissociable groups per gram. Of the proteins thus far studied the prolamins have the smallest number of charges per gram. How far these relations reveal the manner in which reserve proteins are composed of smaller structural units is difficult to say. It is certain, however, that the physicochemical behavior of the smaller reactive molecules, as judged by solubility and crystallizability

TABLE 8

Total number of reactive groups in certain proteins

PROTEIN	PROBABLE MOLECULAR WEIGHT	COMBINING CAPACITY OF 1 GRAM PROTEIN		COMBINING WEIGHT OF THE PROTEIN		COMBINING GROUPS PER MOLECULE	
	*m.w.**	*a*		*1/a*		*a(m.w.)*	
		mols acid $\times 10^{-5}$	*mols base* $\times 10^{-5}$	*gram acid equivalents*	*gram base equivalents*	(+)	(−)
Casein, Hammarsten	192,000	90	183	1,111	546	173	351
Casein, unmodified		60	138	1,667	725	115	265
Serum albumin	45,000	155	155	645	645	70	70
Egg albumin	33,800	80	80	1,250	1,250	27	27
Gelatin	*n*(10,300)	87	57	1,149	1,754	*n*(9)	*n*(6)
Gliadin	*n*(20,700)	34	30	2,941	3,333	*n*(7)	*n*(6)
Zein	*n*(19,400)	0	30	—	3,333	—	*n*(6)

* The minimal molecular weights of these proteins are taken from Lloyd (184), and from Cohn, Hendry and Prentiss (58).

is extraordinarily different from that of the inert precipitates that are found stored in plants and animals.

The charges per molecule of these different proteins, as calculated above, are not in the same order as their charges per unit weight. It might be argued that solubility is determined not by the charge per molecule but by some other function such as the surface density of charge. In order to examine this possibility, the combining capacities of the series of proteins, considered in table 8, have been multiplied by the two thirds power of the molecular weights. This calculation should yield quantities proportional to the surface density of charge, but it does not completely reveal the behavior of proteins. Casein has the largest

number of charges per molecule and per unit of surface, of any of the proteins considered, including the albumins. Despite this the latter are soluble, while casein is very insoluble when uncombined with acid or base. Accordingly we must consider the distribution of charges rather than their total number in relating the physical properties of proteins to their chemical behavior.

VI. THE TITRATION OF THE ACID AND BASE COMBINING GROUPS IN PROTEINS. If a smaller amount of acid, or of base, be added to a protein than is required to neutralize all of the reactive groups in the molecule, the acid or base combined with the protein, under the conditions of the experiment, may be measured.[12] Such experiments were made by Bugarszky and Liebermann (37) upon egg albumin in 1898. When the acid or base combined by the protein is plotted against the hydrogen ion concentration[13] of the solution, or its negative logarithm, pH, a curve is obtained which has been called a titration curve, and which may be used in the characterization of proteins.

D'Agostino and Quagliarello (11) were the first to publish the titration curve of a protein. They studied serum albumin, but only over a restricted range and attempted to estimate the dissociation constants of groups in the protein from the slope of the titration curve. Their measurements together with those of other investigators have been plotted in figure 2. There can be no doubt that essentially the same curve may be drawn through the experimental points of D'Agostino and Quagliarello, of Manabe andMatula (201), of Pauli and Hirschfeld (267), of Pauli and Oden (270), of Hastings,[3] and of Cohn and Berggren.[3] The titration curve must therefore be considered an objective and accurate method of defining the acid and base combining properties of a protein.

In the accompanying figures curves describing a series of proteins of different types have been plotted on the basis of experimental work from numerous laboratories.[8] The method that has been employed in calculating the combined acid and base at the ends of the titration curves has already been explained. The agreement between different measurements made upon different preparations by different investigators is in many cases as satisfactory as in the case of serum albumin.

[12] Combination may be estimated from conductivity (123), (132), (263) as well as from electromotive force measurements. The conductivity method will be considered in connection with ionic mobility of proteins.

[13] In reality the quantity that is generally termed the hydrogen ion concentration is the hydrogen ion activity. Since the former term is in common usage, it is employed in the succeeding sections of this paper.

Measurements made upon casein fall into several categories; those upon normal and those upon modified preparations. The effect of alkali in increasing the acid and the base combining capacity of casein has already been considered. The groups that react at the ends of the curves are apparently not affected by this modification since these segments of the two curves may be constructed by means of the same constants. The experimental points that have been reported by different investigators fall between these curves. Casein thus suffers modification in the most faintly alkaline solutions. Measurements in alkaline solutions upon edestin, serum albumin, and serum globulin (130), (263), (365) are much less concordant than those made in acid solution. Denaturation of these and of other proteins presumably occurs in weakly alkaline solutions that were previously considered innocuous. Whether the denaturation produced by dilute alkali is identical to that produced by acids, by heat, or by alcohol is not certain (42), (104), (240), (265), (326), (329).

The titration curves of different proteins reveal many aspects of their behavior. Thus the curves of egg and of serum albumin are steeper than are those of serum globulin, edestin, and casein. If serum albumin be taken as an example of the most extended titration curve of a protein, that contains both a large number of acid and of basic groups, dissociating with different strengths, then zein may be taken as an example of a protein with a most restricted curve. Indeed zein may be said to have no homogeneous titration curve at all, since it passes into solution only when all of the groups that it possesses have been combined (55).

The globulins, casein and gelatin are relatively insoluble when uncombined with acid or base. The procedure that has generally been employed in the study of gelatin, has prevented a distinction being made between its homogeneous and heterogeneous titration. In the case of the other proteins, the amounts of acid and alkali required to dissolve the protein have been designated by heavy horizontal lines. These have been connected by vertical lines at the hydrogen potentials at which the titration curve is intersected. Within the rectangles so formed, the experimental points represent heterogeneous equilibria as the proteins pass into solution. When these proteins are reprecipitated from acid or alkaline solution, the equilibrium conditions are different, and the slopes of titration curves made under these conditions are always steeper than those here reported.

The slope of the titration curve at any point gives the rate of combination (341) and therefore the relative number of groups dissociating

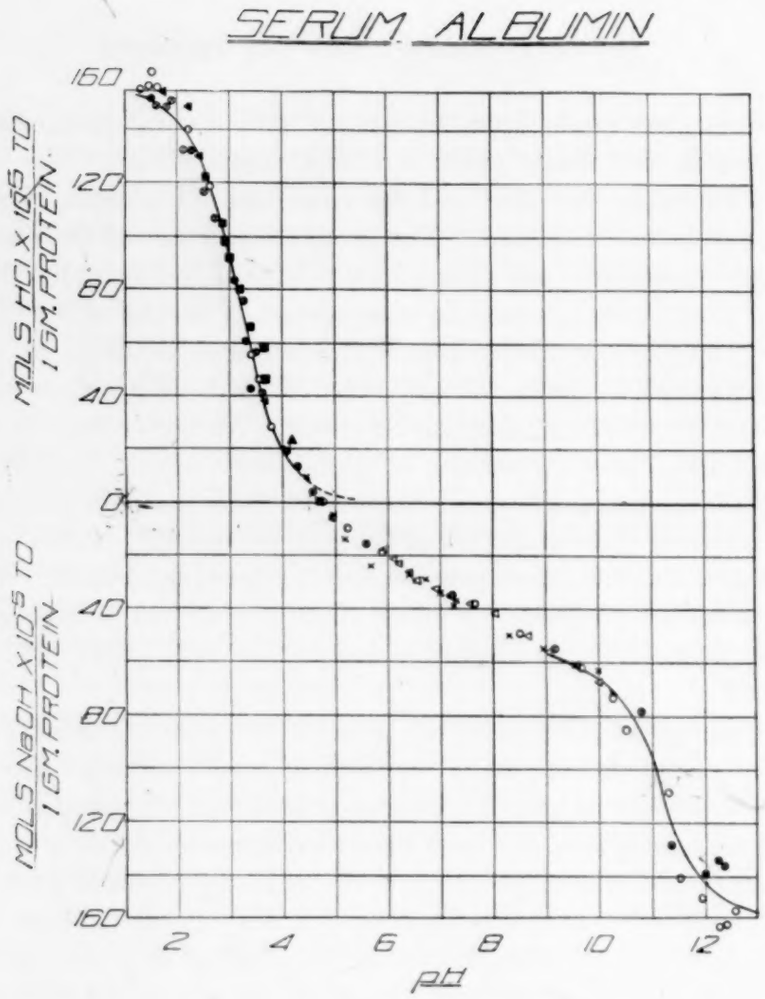


Fig. 2

Investigators	*Symbol*
D'Agostino and Quagliariello (11)	△
Manabe and Matula (201)	●
Pauli and Hirschfeld (267)	▲
Pauli and Odèn (270)	■
Hastings (unpublished data)	×
Cohn and Berggren (unpublished data)	○⊗

D'Agostino and Quagliariello's preparation had a pH of 6.89. It has therefore been assumed that it contained 32×10^{-5} mols of sodium hydroxide per gram. From Pauli and Odèn's measurements 10×10^{-5} mols of acid were subtracted, in order that they might better conform with the other measurements made in Pauli's and in other laboratories. Pauli's measurements (263, p. 114), demonstrating variation with time, have not been included, although they are of the same order.

The curve describing the combination with acid has been constructed on the basis of a single constant, pK = 3.16. Combination at the more alkaline reactions can also be described on the basis of a single constant, pK = 11.10. So many constants would be required to describe the continuous combination in the intervening space that they have not been calculated.

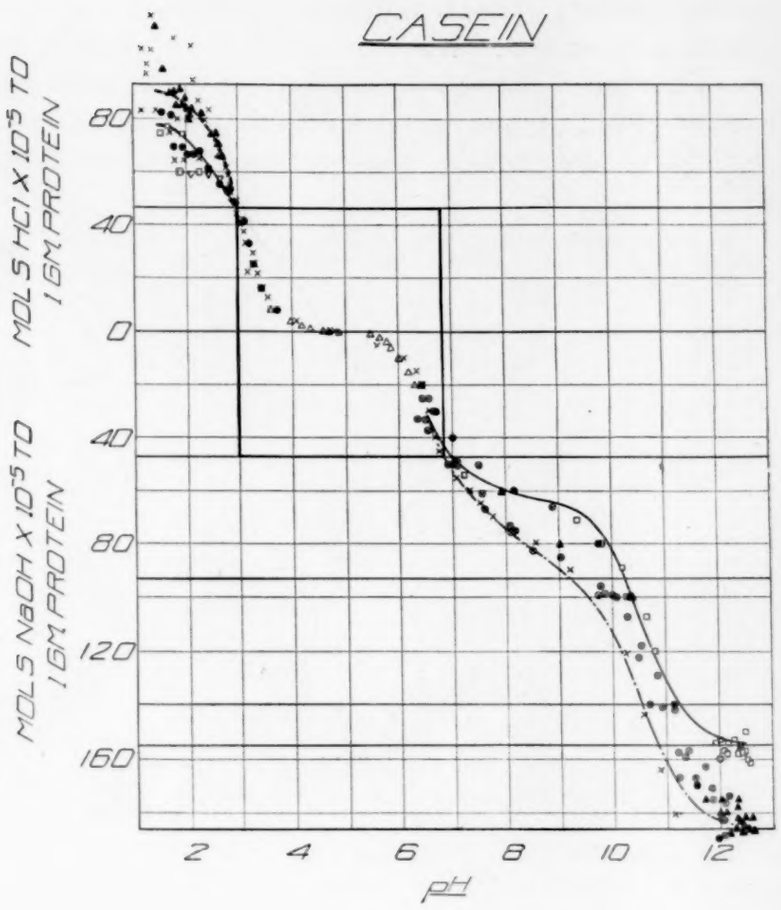


Fig. 3

Investigators	*Symbol* Normal casein	Modified casein
Robertson (296)		⊗
Loeb (190)	●	
Greenberg and Schmidt (90)	⊙	
Hoffman and Gortner (136)		×
Hitchcock (130)	▽	
Cohn and Hendry (57)[3]	△	
Cohn and Berggren (54)[3]	□	▲

Robertson employed potassium hydroxide in his experiments.

Casein passes into solution when one gram is combined with approximately 47×10^{-5} mols of either sodium hydroxide or hydrochloric acid (57)(130). The acid and base combining curves of normal and modified casein have been constructed on the basis of the following dissociation constants; pK = 3.00, 7.10, 8.46, and 10.55 (51).

in the protein molecule. The titrations of equal weights of the different proteins have been plotted. When uncombined proteins are compared, the slopes of the tangents to their titration curves are steeper for albumins than for globulins, and for globulins than for glutelins and prolamins.[14] This observation has suggested that the dissociation of pro-

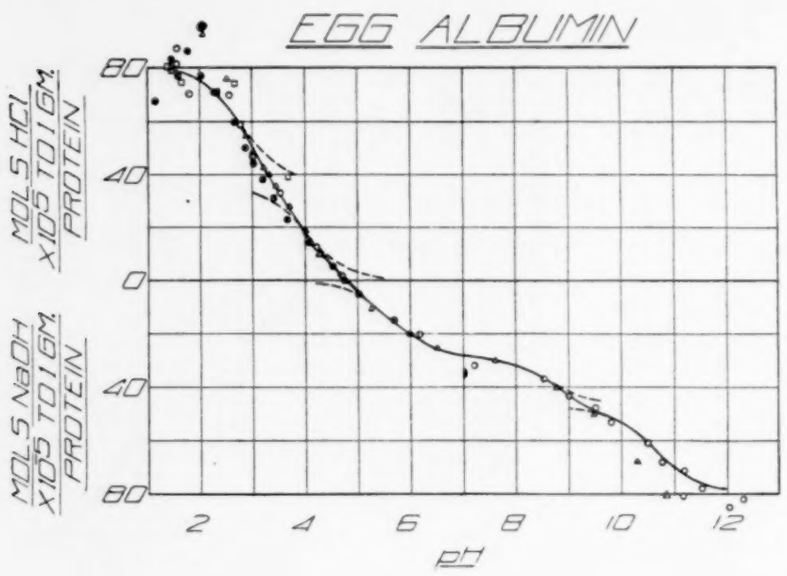


Fig. 4

Investigators	*Symbol*
Bugarszky and Liebermann (37)	□
Loeb (188)	△
Hitchcock (130)	⊗
Cohn and Berggren (unpublished data)	○

Bugarszky and Liebermann's measurements in alkaline solution are omitted, since they are lower and more variable then those of other investigators. The preparation studied by Hitchcock had a pH of 5.67. His data have therefore been corrected for the amount of acid, 15×10^{-5} mols required to bring one gram to the isoelectric point.

The curve has been constructed on the basis of the following five constants; pK = 2.80, 4.00, 5.65, 8.60, and 10.60. When so many constants are involved, an arbitrary element enters into their choice.

[14] The abnormally steep slope of gelatin in all probability depends upon the methods that have been employed in its study. These have resembled the titration of a protein compound, rather than of uncombined protein; personal communication, J. H. Northrup and D. I. Hitchcock. The investigations of Knaggs and Schryver and their colleagues, Biochem. Journ., 1923, xvii, 473; 1924, xviii, 1079, 1085, 1095, which had been overlooked, demonstrate this contention. Gelatin purified by them has a solubility comparable to that reported in table 10.

teins determines those aspects of their behavior upon which their classification depends (49). In the accompanying table the slopes of the titration curves of certain proteins have been referred, not only to their absolute, but also to their molecular weights.

The slopes reported represent, as a first approximation, the number and strength of the dissociating groups in the uncombined protein.

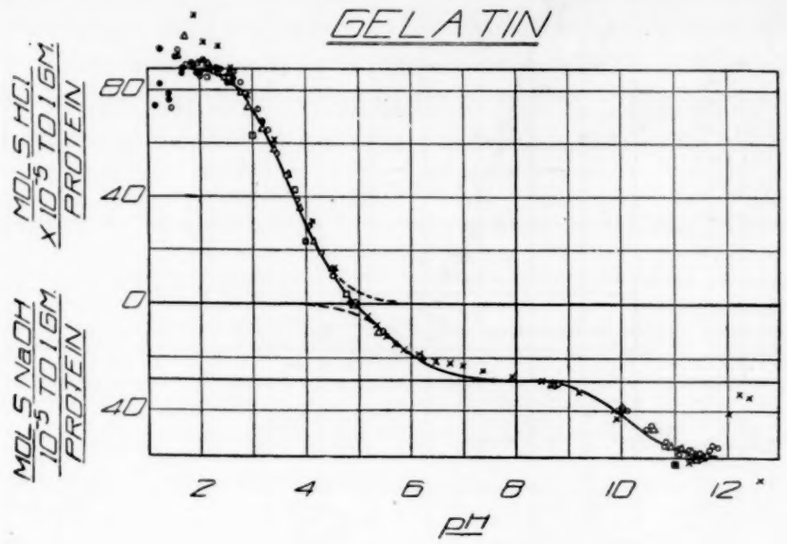


Fig. 5

Investigators	*Symbol*
Manabe and Matula (201)	□
Loeb (195)	△
Hitchcock (128)	●
Hitchcock (128)	○
Hitchcock (130)	⊙
Greenberg and Schmidt (90)	■
Atkin and Douglas (17)	×

The measurements of Blasel and Matula were made at very acid reactions, and are omitted. Those of Lloyd and Mayes have also been omitted, since between pH 2.0 and 10.0 they are, on the whole, consistent with the measurements of Loeb, of Hitchcock, and of Atkin and Douglas, but are not nearly so accurate.

The titration curve of gelatin has been constructed on the basis of the following dissociation constants; pK = 3.7, 5.7, and 10.3, of which the first two were deduced by Atkin and Douglas from their titration curve of gelatin.

The rate of combination increases rapidly, however, as increasing amounts of acid or base are added. Different amounts of acid or base are combined by different proteins in passing into solution. These are also recorded in table 9. The average rate of combination throughout

this range may be roughly estimated from the slope of a diagonal drawn between the points at which solution takes place in acid and in alkali. The greater the vertical dimensions of the rectangles in the figures,

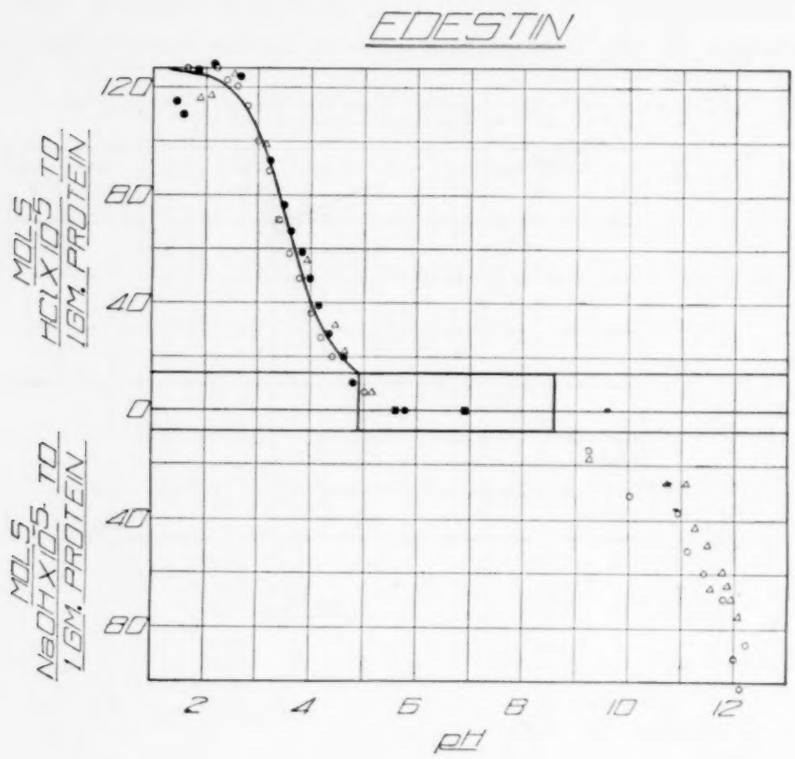


Fig. 6

Investigators	*Symbol*
Hitchcock (127)	○
Hitchcock (130)	●
Kodama (157)	△
Rona and Michaelis (302)	■
Michaelis and Mendelssohn (219)	■

Kodama's preparation, and one of Hitchcock's, were acid. Accordingly 7×10^{-5} mols of acid per gram have been added to these results.

Edestin forms an insoluble hydrochloride containing 7×10^{-5} mols of hydrochloric acid per gram. It dissolves when combined with the same amount of sodium hydroxide and with double the amount of hydrochloric acid (241). The acid titration curve has been drawn on the basis of pK = 3.60.

the greater the amount of acid and base required to dissolve the protein. The greater the horizontal dimensions the greater the difference in strength between the reactive acid and basic groups.

That the combination of proteins with acids and bases was due to their dissociation as amphoteric electrolytes (37), (186), (254), (314) was understood long before the work of D'Agostino and Quagliarello. They attempted to estimate dissociation constants in terms of which they could describe the segment of the dissociation curve of serum albumin that they investigated. The serum albumin was combined with base throughout the range that they studied and therefore their analysis of it in terms of an acid and a basic dissociation has in it an arbitrary element. There is no reason to suppose that a definite number of acid or of basic groups was dissociating in their studies. The total number of groups dissociating at any hydrogen potential is graphically represented,

TABLE 9

The slopes of the titration curves of certain proteins in the neighborhood of their isoelectric points

PROTEIN	SLOPE OF TANGENT TO TITRATION CURVE AT ISOLECTRIC POINT — Combination per pH unit		BASE REQUIRED TO DISSOLVE PROTEIN		ACID REQUIRED TO DISSOLVE PROTEIN	
	mols per gram $\times 10^{-5}$	*mols per mol*	*mols per gram* $\times 10^{-5}$	*mols per mol*	*mols per gram* $\times 10^{-5}$	*mols per mol*
Serum albumin	28†	12.6				
Egg albumin	22†	7.4				
Gelatin	18†[14]					
Hemoglobin	12*	6.0				
Edestin			7 (241)	4	14 (241)	8
Serum globulin	5†	4.0	7 (58)	6	14 (101)	12
Casein	3†	5.8	47 (57)	90	47 (130)	90

† Estimated from titration curves.

* (111).

if we plot the hydrogen potential against the equivalent rather than the absolute weight of the protein, employing for the equivalent weights the values calculated in table 8. The rate of combination with acid or base, and therefore the number of equivalents of approximately the same strength, is greatest for most proteins in very acid and very alkaline solutions, and is smallest and most variable in precisely those ranges which have the greatest physiological significance.

VII. THE DISSOCIATION OF THE ACID AND BASE COMBINING GROUPS IN PROTEINS. The dissociation of amphoteric electrolytes has repeatedly been considered from a theoretical standpoint. Winkelblech (361) and Walker (352) showed that the mass law, which Ostwald had applied to the conductivity of weak acids and weak bases in the form of his dilution law, could also be applied to the behavior of the amino acids.

They studied a number of amino acids by the conductivity method and estimated their dissociation constants. These are recorded in most tables of conductivity and ionization constants (23), (46), (210), (312), and the conclusion has generally been drawn from them that the amino acids are both extremely weak acids and extremely weak bases.

Doubt has arisen as to the interpretation put upon these measurements. Bredig (32), and later Adams (7) and Bjerrum (23) suggested that amino acids exist for the most part as internal salts (Zwitterionen). Bjerrum has, therefore, come to the conclusion that the constants, which earlier workers believed represented the dissociation of acid groups, were really the hydrolysis constants of the basic groups, and conversely that the constants previously considered as representing the dissociation of basic groups were the hydrolysis constants of the acid groups. The acceptance of this view involves no change in the actual values of the constants, but according to it the amino acids could no longer be considered very weak acids and bases.

Dissociation of weak uni-univalent electrolytes. The titration of weak electrolytes with strong electrolytes has also been described in terms of the mass law. The acid dissociation of an amphoteric electrolyte (HPOH) into a hydrogen ion (H^+) and its characteristic anion (POH^-) may be written:

$$\frac{(H^+)\ (POH^-)}{(HPOH)} = K \tag{8}$$

The behavior of a weak acid is accurately described by a mass law equation of this type, but the salt into which even the weakest electrolyte is transformed, upon neutralization by base, must be considered a strong electrolyte (173). Lewis (176) has substituted for the concept of varying dissociation, that of varying activity,[15] and the work of Bjerrum (22) and of Debye and Hückel (69), (236), renders it probable that strong electrolytes are completely dissociated. The assumption is therefore generally made that, although a weak acid (HPOH) dissociates to a very small extent, the salt (BPOH) formed when the acid is neutralized by a base (BOH) dissociates completely. The quantity formerly considered the concentration of the dissociated anion, and now termed its activity,

[15] The mass law was first applied to electrolytes in the belief that their abnormally high osmotic pressures were due to their partial dissociation into ions. In this development no account was taken of the electrical charges of the ions. These result in interionic forces which in the case of strong electrolytes makes a simple statistical treatment of the number of ions and molecules inadequate.

is therefore given by the amount of base present, multiplied by the activity coefficient γ of the ion. This is generally expressed by the relation:

$$(POH^-) = (BPOH)\gamma \tag{9}$$

Substitution in equation (8) for the anion (POH^-) of its value in equation (9) yields the relation:

$$\frac{(BPOH)\gamma}{(HPOH)} = \frac{K}{(H^+)} \tag{10}$$

Certain implications of this equation, such as the fact that the dissociation constant K is equal to the hydrogen ion concentration, when one-half of the acid has been neutralized by base, were first noted by Henderson (116) (117) and Washburn (356), and have repeatedly been demonstrated. If the hydrogen ion concentration be expressed as its negative logarithm, pH, and the dissociation constant by its negative logarithm, pK, equation (10) may be rewritten in the form introduced by Hasselbalch (110), and generally known as the Henderson-Hasselbalch equation:

$$pH^+ = pK + \log \frac{(BPOH)\gamma}{(HPOH)} \tag{11}$$

The comparable equations for the basic dissociation are:

$$\frac{(HP^+)\ (OH^-)}{(HPOH)} = K \tag{12}$$

and

$$pOH^- = pK + \log \frac{(HPA)\gamma}{(HPOH)} \tag{13}$$

The dissociation of water into a hydrogen and a hydroxyl ion, according to the equation:

$$(H^+)\ (OH^-) = Kw \tag{14}$$

makes it possible to express basic as well as acid dissociation in terms of the hydrogen ion. The negative logarithm of the dissociation constant of water, pKw, is equal to the sum of the hydrogen and hydroxyl potentials.

$$pH^+ + pOH^- = pKw \tag{15}$$

Combination of equations (13) and (15) yields:

$$pH^+ = pKw - pK - \log \frac{(HPA)}{(HPOH)} \tag{16}$$

An acid dissociation thus differs from a basic dissociation, as judged by an equation of this type only by a change of sign, and the inclusion of the dissociation constant of water. As a result, the constants determined by the titration of weak acids or bases, by strong bases or acids may theoretically represent either acid or basic dissociation. The valid objections that Bjerrum has raised to considering amino acids as weak acids and bases renders it desirable tentatively to follow the practice introduced by Levene and Simms (171) in their study of polypeptid dissociation, of merely recording the constants as pK_1, pK_2, pK_3, . . . pK_n, without indicating whether they represent acid or basic dissociation. Provided the activity coefficients are equal to unity, these constants indicate the hydrogen potentials at which neutralization has been half completed.

D'Agostino and Quagliarello (11) applied equations of this type to the colorimetric titration of phosphoric acid, glycine and serum albumin. The electromotive force measurements of Walpole (353) upon systems containing acetic acid and sodium hydroxide, of Sørensen (317) upon similar systems containing phosphoric, boric, and citric acids and glycine, the measurements of Walbum (351), of Tague (335), of Eckweiler, Noyes, and Falk (76), and of Harris (107) upon glycine and upon other amino acids make it possible to test the derived equations experimentally. That these hold for dilute solutions of weak uni-univalent electrolytes as a first approximation has often been demonstrated (11), (46), (118), (318), (355).

In the study of dilute solutions of univalent acids, bases, or ampholytes, the assumption has generally been made that the activity coefficient is so nearly equal to unity that it can be omitted. Walpole's data for mixtures of acetic acid and sodium acetate, and Sørensen's data for mixtures of phosphate, demonstrate that this is only justifiable as a first approximation. Johnston (153) and Seyler and Lloyd (313) have shown that consistent values of the ratio of the first to the second dissociation constant can be obtained for mixtures of carbonic acid and sodium carbonate only if very much smaller values are taken for the activity coefficient of the bivalent anion γ_2 than for the univalent anion γ_1. Since the activity coefficient is a function of the valencies of the ions in solution (33), (69), (176), it follows that this factor should not be omitted in the consideration of polyvalent acids, bases or ampholytes.

Dissociation of weak polyvalent electrolytes. The polyvalent character of the proteins complicates the consideration of their dissociation, for the

equations defining polyvalent dissociation contain not one dissociation constant and activity coefficient, but a series of dissociation consants and of activity coefficients (24), (95), (120), (211). Moreover no satisfactory information is available regarding the activity coefficients of protein ions of different valence.

Let us first consider the acid dissociation of a unidivalent ampholyte (H_2POH). Such a substance can form both a univalent and a divalent anion:

$$(H_2POH)K_1 = (H^+)\,(HPOH^-) \tag{17}$$

$$(H_2POH)K_1K_2 = (H^+)^2(POH^=) \tag{18}$$

and if it be titrated with a base, BOH, two compounds (BHPOH) and (B_2POH) result. The distribution of the base between them will, of course, depend upon the reaction, and upon the relative strengths of the dissociation constants. It can always be defined in terms of these compounds by the relation:

$$(BOH) = (BHPOH) + 2(B_2POH) \tag{19}$$

Since these compounds may be considered strong electrolytes, we may substitute for them the ratio of the activity of the ions to their activity coefficients:

$$\gamma_1(BHPOH) = (HPOH^-) \tag{20}$$

$$\gamma_2(B_2POH) = (POH^=) \tag{21}$$

and write:

$$(BOH) = \frac{(HPOH^-)}{\gamma_1} + \frac{2(POH^=)}{\gamma_2} \tag{22}$$

By combining equations (17), (18) and (22), the activities of the ions are eliminated, and we obtain a relation between the hydrogen ion, the base, the partially neutralized acid, its dissociation constants, and the activity coefficients of the ions.

$$(BOH) = \frac{(H_2POH)K_1}{(H^+)\gamma_1} + \frac{2(H_2POH)K_1K_2}{(H^+)^2\gamma_2} \tag{23}$$

This can also be written:

$$\frac{(BOH)}{(H_2POH)} = \frac{K_1}{(H^+)\gamma_1} + \frac{2K_1K_2}{(H^+)^2\gamma_2} \tag{24}$$

and expanded to describe the dissociation of a unipolyvalent acid containing n reactive groups:

$$\frac{(BOH)}{(H_nPOH)} = \frac{K_1}{(H^+)\gamma_1} + \frac{2K_1K_2}{(H^+)^2\gamma_2} + \frac{3K_1K_2K_3}{(H^+)^3\gamma_3} + \cdots \frac{nK_1K_2K_3 \cdots Kn}{(H^+)^n\gamma n} \tag{25}$$

In this equation there are as many terms on the right hand side of the

equation, as there are reactive acid groups in the molecule. The anions formed by the progressive dissociation of acid groups are considered separately, and their sum related to the progressive neutralization of base.

The larger the number of groups in a polyvalent weak electrolyte, that dissociates with approximately the same strength, the steeper will be the segment of the titration curve describing their neutralization. Under these circumstances a number of terms in the equation, representing a number of equivalents, may appear to have a mean valence and a single dissociation constant.

Linderstrøm-Lang (183) has recently attempted to calculate the mean valence of egg albumin over a restricted range, by means of the Debye-Hückel theory of interionic forces and a different treatment of the mass law. The results to which he comes in this most interesting communication are in good agreement with the known maximum valence of egg albumin, especially when the assumptions that he was forced to make are considered. These included the validity of the Helmholtz theory of the electrical double layer, which he has employed in calculating the surface density of charge on the protein.

The fact that the titration curves of proteins represent the summation of the individual dissociation curves indicates that many protein groups are of nearly the same strength. Adams (7) and Bjerrum (24) have deduced a relation between the dissociation constants of dibasic acids and the distance between their carboxyl groups, according to which the first dissociation constant should be at least four times as great as the second. It has occasionally been suggested that this rule should apply to the proteins. Linderstrøm-Lang has pointed out, however, that as the number of groups increases, "so also an increase takes place in the number of dissociation stages, and since the dissociation constants lie close together, there will be several different types of ions with different valencies determining the mean valency" (183, p. 22).

Apparent dissociation of proteins. Uncertainty regarding the valence type of proteins, and the notion that the groups in the protein molecule might be so situated with respect to each other that the dissociation of each would be independent of the dissociation of the others has led most investigators to attempt to analyze the titration curves of proteins in terms of the summation of the dissociation of univalent acids and bases. Whatever the theoretical justification for such a course, the hydrogen potentials of systems containing proteins and acids, or proteins and bases can often be satisfactorily characterized by means of the

unexpanded Henderson-Hasselbalch equation. The combination of either serum albumin or of edestin with acid can be calculated by means of equation (11) and a single dissociation constant with almost as great accuracy as it can be determined experimentally (figures 2 and 6). Only at the most acid reactions does the rate of combination reveal the inadequacy of the assumption involved in these calculations. Otherwise the agreement is extraordinary, in view of the fact that serum albumin and edestin contain approximately 70 and 74 acid combining groups respectively.

Dissociation constants, in terms of which large parts of the titration curves that have been plotted may be characterized, have been calculated and graphically represented in the figures. There can be no doubt that the experimental points are often adequately described by the calculated curves. When more than a small number of constants were required to represent the measurements, as in the case of the neutral range of serum albumin, the arbitrary element in their choice became so great, that no estimation of their strength was attempted.

It is extraordinary that many of the constants describing the behavior of proteins in relatively alkaline and acid solutions have so nearly the same value. In the titration curves reported pK_1 varied only from 2.8 to 3.7, and pKn from 10.2 to 11.1. These ranges are consistent with the notion that the reactive groups in the protein molecule are derived from the groups of the amino acids of which proteins are composed.

Proteins reveal apparent dissociation constants at neutral reactions that known amino acids do not possess. These constants may find their explanation in the influence of neighboring groups upon the strength of groups of amino acids held in polypeptid linkage (76), (171). Such effects unquestionably occur in other ranges as well. Although it is not yet possible to correlate specific groups in the protein molecule with the specific amino acids that they contain, it is not inconceivable that future investigations will reveal such relations. The results that have been reported thus far are consistent with the conception that the dissociation of proteins depends upon the dissociation of the amino acids of which they are composed.

VIII. The ionic mobility of proteins. Proteins are electrolytes. The ions formed by the dissociation of their compounds accordingly participate in the transport of electricity. The second of Faraday's laws asserts that the amount of electricity transported by an ion is proportional to its equivalent concentration, as judged by its chemical

reactions. From the electrochemical equivalent of a protein, it should therefore be possible to judge of its valency. Three methods have been employed in the study of the role of proteins in the transport of electricity. In one of these, the part played by the protein is calculated from the conductivity of its compounds; in the other two the movement of a protein in an electric field is directly measured, and its electrochemical equivalent deduced from its migration.

Conductance method. The conductivity of a solution depends upon the number of ions into which it dissociates, their valency, and their mobility. By studying a protein compound at successive dilutions, the conductivity at infinite dilution can be estimated by extrapolation. By this procedure the term, that has been supposed to represent the degree of dissociation, and that is related to the activity coefficients of the ions, may be eliminated. Conductivity at infinite dilution should theoretically depend only upon the mobilities of the ions. Provided the mobility of the inorganic ion into which a protein compound dissociates is known, the mobility of the protein ion can be estimated from the conductance at infinite dilution. The conductance of an ion should, as a first approximation, be proportional to its valence. "This must be so in the case of ions which, because of their equal size, meet with an equal frictional resistance in moving through the solution, since the electric force driving them is proportional to the charge upon them" (1, p. 762).

A large number of investigators (8), (9), (92), (101), (168), (197), (261), (262), (269), (270), (296), (299) have studied the conductivities of solutions of proteins at successive dilutions. They have attempted to estimate the mobility, the valency, and the equivalent weights of the protein ions from the conductivities of the protein compounds. Many of these results have been considered by Pauli in his recent book upon the Colloid Chemistry of the Proteins (263) and by Greenberg and Schmidt in their recent paper (92). They all indicate that the apparent valency of the proteins is of a low order. Most estimates suggest a lower apparent valency of the proteins than that calculated from their combining capacity and molecular weights. In these calculations the molecular dimensions of the proteins have, however, not been considered. This evidence confirms that considered in the preceding sections.

Conductivity dilution series of only a few protein compounds have been studied. When the conductivities of two or more protein compounds have been measured, it has been found that the conductance of the protein was greater the greater the amount of acid or of base in the

system. The conductivity method has been employed by a number of investigators (123), (132), (189), (263), to study the progressive combination of a finite concentration of protein with acid or base in much the same way as has the electromotive force method. The progressive change in conductance from the most acid to the most alkaline reactions has however not yet been adequately studied in most of the investigations that have been reported.

Hittorf method. In a recent communication, Greenberg and Schmidt (91) have suggested that in systems containing casein and varying amounts of base, the electrochemical equivalent of the casein multiplied by the amount of base present was a constant. They have once more studied transference in alkaline casein solutions, by means of the deposition on the electrodes, and pointed out the faulty interpretation put upon migration experiments by Robertson (298) and by Haas (96). The probable explanation of the results obtained by these investigators has also been considered by Pauli (263). The data of Greenberg and Schmidt are in agreement with the observations of most investigators in that they "are best explained on the assumption that in these solutions the carriers of the current are alkali metal cations and casein anions" (91 p. 300). Over the range investigated from pH 6.5 to 9.4, the base in the system was doubled, and the electrochemical equivalent halved. Presumably the valence also doubled. As a result Greenberg and Schmidt conclude that "Faraday's law holds for these solutions" (91 p. 292).

Moving boundary method. The velocity of migration of certain proteins, and therefore their transference can be directly observed under suitable experimental conditions, or may be followed by one or another analytical procedure. The movement in an electric field of particles charged with respect to their surrounding medium is termed cataphoresis. From cataphoresis the sign and the average magnitude of the charge can be determined, but not its nature or origin. The movement of the colored hemoglobin ion in an electric field was observed by Picton and Linder in 1892 (279) (280). They deduced the sign of its charge from that of the electrode from which it was repulsed. Later Hardy investigated the migration velocities of serum globulin in acid and alkaline solution. Although he believed that "the particles, since they refract light, must be of much more than molecular dimensions" (101, p. 292), he concluded that their charge was

> due to a real electrotytic dissociation at the surface of the particles, the degree of dissociation depends upon the same factors which in the interaction between

solvee and solvent determine the degree of dissociation of different electrolytes, and it fixes the density of the charge, while the amount of electricity carried per unit of weight is the product of the density and the area of the surface between the particles and the fluid in which they float (101, p. 292).

Hardy did not distinguish between the velocity of migration of a charged particle and a dissolved molecule, since over the range that he studied "the solution changes from milk-white or non-settling suspension stage, to transparency" (101, p. 291). He measured the migration velocity of serum globulin in the presence of acid and alkali. More recently Adolf (10) has studied serum globulin, Michaelis and Airila (212) have studied hemoglobin, and Scott and Svedberg (311) have measured the change in migration velocity of egg albumin at different hydrogen ion concentrations. Laszlo Reiner, working in this laboratory, has also studied the migration velocity of hemoglobin and of egg albumin. His measurements upon the albumin extended from reactions at which the protein was saturated with acid to those at which it was saturated with base. Except in the most acid solutions studied, where Reiner observed slightly greater velocities, these measurements confirm the general magnitude of those of Scott and Svedberg. Reiner observed migration velocities in alkaline solutions as great as those in acid solutions. Moreover, he noted zones in which no appreciable change in velocity occurred. Apparently no additional groups in the protein molecule were dissociating at these reactions. In so far as migration velocities are proportional to ionic mobilities, they reveal the valence of the protein. These zones should therefore yield the relative numbers of groups dissociating at different hydrogen ion concentrations. The shape of migration velocity curves, as of titration curves, indicates that many of the dissociation constants of proteins have approximately the same strength.

The same equations have generally been employed in calculating from migration velocities the charge both of colloidal particles and of protein molecules. These will not be considered here, excepting to note that Debye and Hückel (69), in their study of crystalloidal ions, have applied conceptions similar to those in terms of which McBain and Salmon have explained the very high mobilities of electrolytes of colloidal dimensions:

Colloidal electrolytes are salts in which an ion has been replaced by a heavily hydrated polyvalent micelle that carries an equivalent sum-total of electrical charges and conducts electricity just as well or even better than the simple ion it replaces (205, p. 426).

If the assumption is justified that under the best experimental conditions the migration velocity of a protein is proportional to the number of its charges, then despite the larger experimental errors, such measurements have a great theoretical advantage over conductivity or electromotive force measurements. In the latter, the quantity measured is the activity of the hydrogen ion, of the chloride ion, or of some other ion; conductivity data, even when extrapolated to infinite dilution yield the sum of the conductances of all the ions; whereas the moving boundary method measures the mobility of the protein ion.

IX. The isoelectric points of proteins. The concept of the isoelectric point had its origin in the observation that "the direction of the movement of colloidal particles under the influence of an electric current is determined by their chemical nature" (100, p. 297). Picton and Linder had noted that hemoglobin moved under the influence of a constant electric current (279), (280), and Hardy observed that under similar conditions

> the particles of proteid in a boiled solution of egg-white move with the negative stream if the reaction of the fluid is alkaline; with the positive stream if the reaction is acid (100, p. 304).

Pauli (259), (265) later investigated the direction of migration of undenatured serum albumin in an electric field, and found that it, too, moved toward the anode in an alkaline solution, and toward the cathode in an acid solution. The charge on the protein was negative in alkaline, but positive in acid solution.

Improvements in the technique of determining the direction of migration followed (47), (167). Michaelis was the first investigator to determine accurately the hydrogen ion concentration[13] at which the migration of a protein changed its direction. He studied serum albumin, and found that the change was abrupt (207). It occurred within exceedingly narrow limits. At a hydrogen ion concentration of 2.1×10^{-5} serum albumin migrated toward the anode. At hydrogen ion concentrations smaller than 1.9×10^{-5} it migrated toward the cathode. At 2.0×10^{-5} the protein appeared to be in an isoelectric condition (214), and this hydrogen ion concentration was accordingly termed the isoelectric point.

Dissociation theory has generally been applied to the definition of the isoelectric point of a uni-univalent substance. Under these conditions an expression for the dissociation of the ampholyte is obtained on multiplication of equations (8) and (12) for the dissociation of a weak

acid and a weak base respectively. The constant in equation (8) is here written Ka; in equation (12) Kb.

$$\frac{(HP^+)\ (POH^-)}{(HPOH)\ (HPOH)} = \frac{Ka \cdot Kb}{Kw} \tag{26}$$

The method of deriving this relation involves the elimination of the hydrogen ion concentration. As a result the pure uni-univalent ampholyte can exist at only one hydrogen ion concentration, namely, that at which the ampholyte dissociates to form as many anions as cations. This point has been identified as the point at which migration of protein in an electric field changes in direction, that is, as the so-called isoelectric point. It has been defined by Michaelis as the point at which the cation concentration is equal to the anion concentration:

$$(HP^+) = (POH^-) \tag{27}$$

and is determined by substituting in equation (27) the value of (POH^-) in equation (8) and of (HP^+) in equation (12). We then obtain

$$\frac{Ka\ (HPOH)}{Kb\ (HPOH)} = \frac{(H^+)^2}{Kw} \tag{28}$$

and if we assume that the protein molecule is the same on both sides of the isoelectric point (76) the last equation becomes

$$\text{Isoelectric point} = (H^+) = \sqrt{\frac{Ka}{Kb}}\,Kw \tag{29}$$

These equations define the conditions that exist at the isoelectric points of simple ampholytes. They enabled Michaelis to characterize the isoelectric point of a simple ampholyte as the point (a), at which its dissociation was at a minimum; (b), its anion concentration equal to its cation concentration; and (c), on one side of which it dissociated as an acid, on the other as a base (210).

In the last section we have seen that the migration velocity of a protein may, as a first approximation, be considered proportional to the number of its charges. At the isoelectric point, a protein migrates in neither direction. It must, therefore, possess as many positive as negative charges. The sum of the dissociated acid groups must accordingly be equal to the sum of the dissociated basic groups:

$$(HP^+) + (HP^{++}) + (HP^{+++}) + \cdots (HP^{n+}) = (POH^-) + (POH^{=}) + (POH^{\equiv}) + \cdots (POH^{\iota-}) \tag{30}$$

Levene and Simms (171) have considered the equation that defines the dissociation of polyvalent ampholytes. Their expanded expression for the isoelectric point need not be reproduced. An expression sufficiently exact for present purposes results (163), (171), if the dissociation constants in equation (29) are replaced by a series of dissociation constants.

$$\text{Isoelectric point} = (H^+) = \sqrt{\frac{Ka_1 + Ka_2 + Ka_3 + \cdots Ka_n}{Kb_1 + Kb_2 + Kb_3 + \cdots Kb_n}} \cdot Kw \qquad (31)$$

The concept of the isoelectric point has a two-fold origin. Born of colloidal chemistry, it has acquired a theoretical significance and a mathematical definition in terms of electrolytic dissociation.

Isoelectric points of water soluble proteins. The isoelectric points of those proteins that are soluble in water have been derived both by the cataphoretic method and by methods deduced from electrolytic dissociation theory. The narrow range of hydrogen ion concentrations at which cataphoresis reveals the change in sign of the charge borne by serum albumin has already been alluded to. If the assumption be made that the charge on the protein is due to its dissociation as an amphoteric electrolyte, the addition of salt to a protein solution should change the activity of the protein anions and cations. The exhaustive researches upon egg albumin, carried out by Sørensen and his collaborators, have led them to conclude:

> at hydrogen-ion concentrations which are not situated at or in the neighborhood of the isoelectric point of the ampholyte the capacity to combine with acids
>
> (a) is independent of the ampholyte concentration;
>
> (b) increases with rising concentration of salt;
>
> (c) is positive at hydrogen-ion concentrations superior to that corresponding with the isoelectric reaction of the ampholyte, and
>
> (d) is negative—that is, the ampholyte is combined with surplus base—at hydrogen-ion concentrations inferior to that corresponding with the isoelectric point of the ampholyte (325, p. 161).

These criteria led Sørensen to conclude that "the isoelectric point is the same in all the examined samples of egg-albumin," "is independent of the concentration of egg-albumin," and is situated at a hydrogen ion concentration of $15–16 \times 10^{-6}$. It is also "independent of the concentration of ammonium sulfate, whereas the amount of surplus sulfuric acid naturally increases with increasing concentration of ammonium sulfate" (325, p. 156).

The minimum solubility should coincide with the isoelectric point on the simplest assumptions that can be made. Sørensen studied the crystallizability of egg albumin from solutions containing the protein and ammonium sulfate, and found that the optimum conditions for crystallization, and the minimum solubility coincided with a hydrogen ion concentration of 26.3×10^{-6}N (323). The minimum solubility thus occurred at a hydrogen ion concentration perceptibly more acid than the isoelectric point as determined by the cataphoretic or the "titration curve" method.

Two explanations of this phenomenon may be advanced. Either the compounds of egg albumin containing surplus ammonia are more soluble than those containing surplus sulfate; or both compounds are relatively soluble when compared with the compounds of egg albumin and ammonium sulfate. The latter hypothesis supposes that minimum solubility reveals minimum dissociation, while the former assumes that minimum dissociation coincides with the isoelectric point (171) (210), and that minimum solubility is to be accounted for by the different equivalent combining weights and the different activity coefficients of protein ions.

Hemoglobin is less soluble than the albumins. Whereas albumins can only be crystallized from concentrated solutions of neutral salts in the neighborhood of their isoelectric points (138), (140), (321), hemoglobin under comparable conditions crystallizes from water. Hemoglobin is, however, sufficiently soluble at its isoelectric point to enable cataphoretic measurements to be made. At a hydrogen ion concentration of 1.2×10^{-7}N hemoglobin migrates toward the anode in an electric field. At a hydrogen ion concentration of 2.4×10^{-7}N migration is cathodal. The hydrogen ion concentration at which the direction of migration changes is therefore approximately 1.8×10^{-7} N (80), (213), (216).

In the neighborhood of its isoelectric point, hemoglobin crystallizes from concentrated solutions (80), (115). The precise point at which the solubility of hemoglobin is at a minimum has not been determined, but one method of crystallization depends upon the transformation of reduced into oxyhemoglobin (74). Oxygenation changes the strength of the acid dissociation of hemoglobin (119), (257), and presumably also the isoelectric point (112), (331). The oxygen combining affinity of this protein, and certain related properties, are at a minimum at a hydrogen ion concentration more acid than the isoelectric point determined by cataphoresis (6), (111).

Isoelectric points of salt soluble proteins. Globulins are by classification insoluble in water, but soluble in neutral salt solution. To this definition should be appended the phrase "in the neighborhood of their isoelectric points." When dissolved by acids or by bases, globulins are relatively soluble and migrate in an electric field. When they are dissolved in salt solution in the neighborhood of their isoelectric points, however, they do not move with the current in either direction (38), (224). Since the zone in which they remain motionless is relatively wide, the cataphoretic method is not readily applicable to them.

Globulins are dissolved by neutral salts over a considerable range of hydrogen ion concentrations. Practice has, therefore, consisted in reducing this range by the removal of salt, and then either in noting the point of maximum flocculation of the globulin, or the limiting hydrogen ion concentrations at which migration occurred in an electric field. Michaelis has estimated that the isoelectric point of serum globulin determined by the cataphoretic method coincided with a hydrogen ion concentration of approximately 3×10^{-6}N (38), (224), and that the optimum condition under which this protein flocks coincides with a slightly more acid reaction of 3.6×10^{-6}N. Adolf (10) believes, however, that the isoelectric point of this globulin cannot be determined by its flocculation maximum, since the latter is dependent upon the quantitative relations between the globulin and the salts in the solution, and upon the nature of these salts.

The vegetable globulin, edestin, as Osborne demonstrated in 1902 (241) is not only relatively insoluble itself, but forms a relatively insoluble acid compound. This is unquestionably one of the causes of the wide precipitation zone of this protein. Rona and Michaelis estimated, from the maximum precipitation of edestin in a series of phosphate buffers, that its isoelectric point corresponded with a hydrogen ion concentration of 1.3×10^{-7}N. (302). Later Michaelis and Mendelssohn using acetate buffers in which to study the optimum flocculation of edestin believed that the isoelectric point coincided with a hydrogen ion concentration of 2.5×10^{-5}N. (219). Hitchcock noted that the point of maximum precipitation of the preparation of edestin that he studied "seemed to vary with the buffer used" (127, p. 598). Apparently it has not been possible thus far to differentiate between neutral edestin and the insoluble compounds that this, and presumably other globulins, form with acids. It would seem as though Osborne's analytical procedure was less equivocal than the physicochemical methods that have been applied to globulins without adequate consideration of their special chemical characteristics.

Edestin is possessed of but a small number of acid and basic groups that dissociate in the neighborhood of its isoelectric point. Osborne's measurements indicate that on the basis of a molecular weight of 58,000 edestin contains but four groups which can combine base in the neighborhood of the isoelectric point to form a soluble compound, and four which combine with acid to form the insoluble compound referred to above. A combination of acid with four other groups transforms the insoluble into a soluble hydrochloride (241). In all, therefore, only twelve groups dissociate to any appreciable extent from pH 4.5 to 8.5. In this respect, a globulin like edestin behaves in a manner comparable though not identical with such monoamino monocarboxylic acids as glycine, alanine, and leucine. "Between pH 4.5 and 8.0, the above three amino-acids are undissociated, exert no buffer effect, and behave in solution much as non-electrolytes. If we calculated the isoelectric point of such a substance, the value has no practical significance. There is in reality an isoelectric *range* from pH 4.5 to 8.0, in which there is no change in properties either physically or from the standpoint of ionization" (171, p. 813). In the case of the globulins there are, as the formation of insoluble compounds demonstrates, a certain number of reactive groups but not enough to carry these large molecules into solution.

Isoelectric points of slightly soluble proteins. One of the prolamins, zein furnishes us with an example of a protein which does not behave like an amphoteric electrolyte, and which cannot be said to possess an isoelectric point. Zein does not combine with acid. As a result it does not dissolve in dilute acid solutions more than it does in water (55).[16] Moreover, zein possesses so small a number of base combining groups that it does not pass into solution until nearly all of them are combined, or at a hydrogen ion concentration smaller than 10^{-10}N. It must possess certain groups which dissociate at less alkaline reactions, since it combines with alkali to some slight extent. Neutralization of these groups does not, however, result in the formation of soluble compounds.

Gliadin, another prolamin, behaves in a manner comparable to zein at neutral and alkaline reactions, but differs from zein in possessing groups which dissociate in acid solution. As a result, gliadin is soluble when combined with either sufficient acid or alkali, but insoluble over

[16] Hoffman and Gortner (136) report a titration curve of zein and acid. Experiments in this laboratory upon two zein preparations, one of which was supplied by T. B. Osborne, failed to reveal any acid combining properties.

the whole range of reactions extending from 10^{-3} to 10^{-10}N. Wood and Hardy estimated that the isoelectric point of gluten, which they believed to be identical with that of gliadin, occurred at hydrogen ion concentrations between 10^{-7} and 10^{-8}N (364). Rona and Michaelis placed the isoelectric point at a still more alkaline reaction, namely 5.9×10^{-10}N but note that:

> Es ist bemerkenswert, dass bei den Reihenversuchen mit zunehmenden HCl-Mengen bei höheren HCl-Konzentrationen eine neuerliche Fällung auftritt, nachdem bereits, in den vorhergehenden Röhrchen vollständig klare Lösung zu beobachten war. Dies ist auf die Bildung eines in Überschuss von HCl unlöslichen Hydrochlorates zurückzuführen. Die Zurückdrängung der Dissoziation in diesem Falle ist auch aus den Überführungsversuchen ersichtlich (302, p. 198).

Eto and Tague have come to the conclusion that the isoelectric point of gliadin occurs at a less alkaline reaction, namely pH 6.6 (79), or 6.5 (336). But the methods that they have employed are subject to the criticisms that have been advanced in the case of edestin.

These conflicting results render it certain that the isoelectric point is not easily determined in the case of proteins which form a series of insoluble compounds. The interpretation of their behavior demands a knowledge not only of the reaction at which the neutral protein exists, but of the reactions and compositions of the several insoluble compounds.

Proteins like gelatin and casein, although they are very nearly as insoluble as the prolamins, have very much narrower precipitation zones. The isoelectric point of gelatin has been determined by Michaelis and Grineff, and coincides with a hydrogen ion concentration of 2×10^{-5}N (218). The researches of Loeb have demonstrated that many of the physical properties of this protein are at a minimum in this neighborhood (195). Gelatin, as we have seen, contains very many more acid combining groups than gliadin, and has a more acid isoelectric point. It does not, however, contain very many more base combining groups, revealing only six on the basis of a minimal molecular weight of 10,300 (58). Of these one-half, or three, dissociate at the alkaline reactions at which gliadin and zein pass into solution, and only three dissociate in the neighborhood of the isoelectric point. There is thus a considerable zone, between pH 7.5 and 8.5, at which few additional groups dissociate. "In order to explain certain experiments on the swelling of gelatin, together with other data collected from the literature, Wilson and Kern . . . suggested that gelatin exists in two forms having different isoelectric points. At temperatures above 35°C. they assumed that gelatin exists only in a

"sol form" having its isoelectric point at pH 7.7, while at lower temperatures it exists as mixtures of this with the "gel form" having its isoelectric point at pH 4.7" (133, p. 457). Hitchcock has demonstrated that the contention that gelatin has two isoelectric points is untenable. It is more probable that the critical zone which has been observed in the physical properties of gelatin coincides with the region in which there occurs no appreciable combination with base. Such a zone occurs in gelatin under precisely these conditions, and is the more noteworthy because in very few proteins does the titration curve show such distinct zones. The dissociation of new groups becomes negligible in the neighborhood of pH 7.7 (see fig. 5). Related to this in all probability are the observations of Wilson and Kern that the swelling of gelatin passes through two minima, of Mathews, and of Higley that the absorption spectrum of gelatin has "minimum values for the wave length of maximum absorption in the ultraviolet at pH 4.68 and 7.66" (360, p. 3140). The presence of salt may be expected to affect the activity of the protein ions that exists at these reactions. Similar minima and maxima have been observed in the case of other proteins (56).

Of the difficultly soluble proteins that have been considered, casein possesses the largest number of acid and of basic groups. Moreover, it possesses a larger number of groups that dissociate in the neighborhood of its isoelectric point, than the other proteins of this class, and indeed a larger number than many globulins. Its slight solubility in the neighborhood of its isoelectric point presumably depends upon its high molecular weight, and therefore upon the low surface density of the charge.

Michaelis and Pechstein have shown that the direction of migration of casein changes in the zone limited by hydrogen ion concentrations of 1.3 and 4.9 $\times$ 10^{-5}N and that the optimum reaction for its flocculation coincides with a hydrogen ion concentration of 2.4 $\times$ 10^{-5}N (222). The precipitation of this protein at this point is remarkable, and its purification depended upon its relative insolubility under these conditions long before the theory of the isoelectric point had been developed. Even in this case, Michaelis has shown that the presence of salts of high valence shifts the point of maximum precipitation (226).

The relations between the isoelectric points, or zones, of slightly soluble proteins, and the acid and basic groups that they possess, have been tabulated both on the basis of their absolute and of their molecular weights. These results demonstrate that the dissociation of proteins determines the extent of their precipitation zones, and their solubility when precipitated.

X. THE SOLUBILITY OF PROTEINS IN WATER. Even the most insoluble protein that has been studied thus far dissolves in water to some extent. Solubility in water has generally been studied by bringing a relatively insoluble protein to its isoelectric point and allowing it to precipitate. There remain in solution varying amounts of soluble protein, which represent not only the protein, but the compounds of the protein that are in equilibrium with the excess of acid or alkali which always exists at the isoelectric point (unless the isoelectric point corresponds with the absolute neutrality of water) and with the salt that has accumulated in the course of the neutralization and precipitation. If the precipitate is repeatedly triturated with water, the soluble compounds of the proteins are removed, together with the compounds of any other proteins that are present, unless they have the same isoelectric points, and the same or lower solubilities than the protein that is being purified. After salt, acid or alkali, and the soluble protein compounds that constitute impurities have been removed by the successive fractions of water with which the precipitate is brought into equilibrium, only the isolated purified protein remains, and its solubility becomes constant. The protein then dissolves in water to a constant and characteristic extent, even when the amount of protein precipitate with which the solution is in equilibrium is varied within wide limits. When solubility is independent of the amount of the precipitate, simple heterogeneous equilibrium may be supposed to exist between the solution and a single saturating body. The solubility in water of a pure protein may therefore be considered as a fundamental, physicochemical constant which may be used in identifying and in classifying proteins.

The solubility in water of a number of proteins is recorded in table 10. These solubilities are exceedingly small, when they are expressed as grams of protein in a liter of water. The molecular concentrations of these saturated solutions are obtained by dividing the weights dissolved by the molecular weights of the respective proteins. This has been done in the third column of the table. The proteins thus far studied range from zein to hemoglobin, that is to say, from a protein that is insoluble throughout the neutral range to a protein which is highly dissociated at its isoelectric point, whose precipitation zone is very narrow, and whose solubility in water is lower only than that of the water soluble albumins.

The solubility of zein, casein, and serum globulin has been determined by saturating successive amounts of water with them. The solubility of casein and zein, was independent of the amount of precipitate with

which the solution was in heterogeneous equilibrium. In serum globulin, however, there was a "persistent higher solubility in the flasks containing most precipitate" (50, p. 715). This observation is related to that of Sørensen, that the solubility of the globulins in water, as in salt solution, cannot be explained on the basis of a single saturating body. (319).

The solubilities of hemoglobin reported by Landsteiner and Heidelberger (166) vary considerably, since they were seeking not absolute,

TABLE 10

The solubility of certain proteins in water

PROTEIN	SOLUBILITY OF PROTEIN IN WATER		HYDROGEN ION CONCENTRATION OF PROTEIN[13]	
			Uncombined	Isoelectric
	grams per liter	*mols* $\times 10^{-4}$	*mols* $\times 10^{-6}$	*mols* $\times 10^{-6}$
Zein.....(55)	0.054		2	
Serum globulin* .....(50) (224)	0.076	0.94		3.6
Casein.....(50) (57) (222)	0.110	0.58	13	24.0
Gelatin.....(218)[3,14]	0.20		3	20.0
Gelatin.....(31)	1.2			
Hemoglobin, horse .....(166)† (216)	8.0	160		0.15
Hemoglobin, dog .....(166)†	23.8	476		

* Different gobulin fractions have different solubilities in water (319).

† The lowest values have been taken.

but only relative, results. The oxyhemoglobin crystals were, therefore, not triturated with large volumes of water, and the solubilities may be high, although they unquestionably represent the order of magnitude. Bradford's value for the solubility of gelatin in water was also not obtained by saturating water with gelatin. It represents a strength of solution which, upon being brought to a lower temperature, "continued perfectly clear with no trace of opalescence" (31, p. 557). An isoelectric gelatin solution studied by J. L. Hendry in this laboratory (for which we are indebted to D. I. Hitchcock) had a solubility of this order before it was washed with water.[14]

The solubilities that have been measured by saturating water with respect to protein are so low that it seemed possible that equilibrium was not reached between the precipitate and the solution. Accordingly attempts have been made to approach equilibrium by precipitating, rather than dissolving proteins. This was accomplished by concentrating in vacuo saturated solutions of pure proteins, filtering off the precipitate that appeared upon concentration, and determining solubility by measuring the concentration of protein in the filtrate. Experiments conducted in this manner upon casein and serum globulin indicate that the results in table 10 represent solubility in systems that were in equilibrium.

The concentration of a solution of pure protein must be the sum of the concentration of the undissociated protein molecules which are in heterogeneous equilibrium with the protein precipitate, and of the concentrations of the ions into which the protein dissociates. At the isoelectric point the sum of the dissociated acid groups must be equal to the sum of the dissociated basic groups.

$$(HP^{+}) + (HP^{++}) + (HP^{+++}) + \cdots (HP^{n+}) = (POH^{-}) + (POH^{=}) + (POH^{=}) + \cdots (POH^{n-}) \quad (30)$$

With the removal of all ions other than those derived from the dissociation of the protein, we may express the concentrations of protein anions and cations in terms of the concentrations of hydrogen ions and hydroxyl ions, and the condition for the neutrality of the solution. The only positively charged ions in a pure solution of uncombined protein are the protein cations and the hydrogen ions, and the only negatively charged ions are the protein anions and the hydroxyl ions. Since the sum of the positive ions in the solution must be equal to the sum of the negative ions, we may therefore write:

$$(H^{+}) + (HP^{+}) + (HP^{++}) + (HP^{+++}) + \cdots (HP^{n+}) = (OH^{-}) + (POH^{-}) + (POH^{=}) + (POH^{=}) + \cdots (POH^{n-}) \quad (32)$$

The dissociated ions of the dissolved protein give a hydrogen ion concentration to water which is also a characteristic of each protein (50), although this hydrogen ion concentration need not be identical with the isoelectric point of the protein (171), and can only be so, provided the dissociation of the protein is such that the hydrogen ion concentration of its solution is precisely equal to the hydroxyl ion concentration. Only in this case will equation (30) be identical with equation (32), and the hydrogen ion concentration of a solution of a pure ampholyte be identical with

the hydrogen ion concentration that is characteristic of its isoelectric point.

The hydrogen ion concentrations of certain of the solutions of pure proteins whose solubilities have been measured are tabulated. From them the difference in the concentration of protein anions and cations may be calculated. This is equal to the difference in the hydrogen and the hydroxyl ion. When the hydrogen ion concentration produced by the dissociation of the pure protein is greater than 10^{-5}N as in most of the proteins studied, the hydroxyl ion concentration is smaller than 10^{-8}N. The hydroxyl ion concentration is, therefore, negligible, and the difference between the sum of the anions and the sum of the cations may be considered approximately equal to the hydrogen ion concentration. This value is also the smallest concentration that can be ascribed to the anion concentration, and represents the smallest possible dissociation. The change in hydrogen ion concention that results from the purification of isoelectric protein from the excess of acid or alkali with which it is in equilibrium will, therefore, be the greater (a), the further the isoelectric point is removed from the neutral point; (b), the smaller the solubility of the protein; and (c), the weaker its dissociation.

XI. THE SOLUBILITY OF PROTEINS IN DILUTE ACID, BASE, OR SALT SOLUTIONS. Certain proteins which are relatively insoluble in water become very soluble in the presence of acids, bases or salts.

Solubility in systems in which proteins are the saturating bodies. When sodium hydroxide is added to casein, an amount passes into solution which is proportional to the base added (57). When equilibrium is established in such systems, solubility is independent of the amount of casein precipitate. This has been demonstrated by varying the amount of the casein precipitate, and also by saturating with casein successive quantities of dilute sodium hydroxide. The same concentration of casein always dissolved in a sodium hydroxide solution of the same concentration. A heterogeneous equilibrium between the solvent and a single saturating body must therefore be established in this case. Under these circumstances the solubility, S, may be put equal to the sum of the solubility of the undissociated protein molecule, Su, and the sum of the concentrations of the anions, provided systems are studied sufficiently far from the isoelectric point for the cation concentration to be negligible.

$$S = Su + (POH^{-}) + (POH^{=}) + (POH^{\equiv}) + \cdots (POH^{n-}) \qquad (33)$$

Studies upon casein have indicated that this protein passes into solution

as though it were dissociating as a divalent acid. The concentration of the divalent protein anions in the last equation may therefore be put equal to the product of the concentration of the undissociated molecule, and the dissociation constants, Ka_1 and Ka_2, divided by the square of the hydrogen ion concentration, and rewritten:

$$S = Su + \frac{Su \cdot Ka_1 \cdot Ka_2}{(H^+)^2} \tag{34}$$

The only variables in this equation are the solubility of the casein and the hydrogen ion concentration. Both of these quantities have been determined in systems containing casein and different amounts of sodium hydroxide (57). A comparable study upon an amino acid, tyrosine, has now been reported by Hitchcock (134), and the theory of the acid base equilibrium in various two phase systems has been considered by Murray (231).

The solubility of proteins in systems containing sodium hydroxide yield not only the dissociation constants of the protein, and the solubility of the molecule, but also the equivalent combining weight of the protein. The latter results if the concentration of the anions be divided by the concentration of alkali. The equivalent combining weight of casein as estimated by this method varied from 2,096 to 2,166 grams per mol sodium hydroxide. Since the probable molecular weight of casein is 192,000, approximately 90 acid groups in the casein molecule must be involved in its passage into solution. It is extraordinary that despite this large number of groups, casein appears to behave as though it were a divalent acid.

Solubility in systems in which protein compounds are the saturating bodies. If instead of sodium hydroxide, calcium hydroxide be added to casein, the protein also passes into solution. Solubility in systems containing casein and calcium hydroxide is not, however, independent of the amount of the saturating body. More calcium hydroxide must be added the more casein is present before solubility appreciably increases, since the casein forms an insoluble, as well as a soluble, calcium compound. The uncombined casein must therefore first be transformed into an insoluble calcium compound. This is then changed into a soluble compound, by the dissociation of other base combining groups. Solubility increases in such systems upon the addition of more alkali in the same way that it does in systems containing casein and sodium hydroxide. The former case remains the more complicated, however, since two saturating bodies may be present, namely, the casein molecule

and its insoluble calcium compound. The degrees of freedom of the system, as defined by the phase rule, are thus increased. Both the calcium and the casein being present in more than one phase, the solubility of casein in solutions of calcium hydroxide is not independent of the amount of casein in the system.

Many proteins combine with acids and bases to form insoluble as well as soluble compounds. Osborne (241) showed that edestin combined with 7×10^{-5} mols of hydrochloric acid per gram, to form an insoluble hydrochloride, and that it formed a soluble compound with twice this amount of acid. The precipitates of globulins and of other proteins that separate from acid solutions upon the addition of small amounts of neutral salts are also presumably compounds of the proteins and acids (203), (244). Under certain circumstances soluble proteins form insoluble compounds with salts of the heavy metals (204), (249), (268). Equilibria in such systems have been interpreted in terms of the phase rule by Galeotti (86) on the assumption that the protein salt and water are distributed between the phases.

Certain of the prolamins have very wide precipitation zones. Zein and gliadin do not pass into solution until very alkaline reactions are reached but nevertheless combine base at neutral reactions. They appear to form a series of insoluble compounds. Van Slyke and Hart (346) and Van Slyke and Van Slyke (347) have studied the insoluble compounds that casein forms with smaller amounts of acid than are required to carry it into solution. Fibrin, according to Bosworth (27), forms a soluble hydrochloride and a soluble sodium compound, but like casein, this protein forms an insoluble calcium compound that contains twice as many equivalents of base as the soluble sodium compound. Fibrin would thus appear to combine with very small amounts of base in molecular rather than equivalent proportions. Hardy has made a similar observation concerning serum globulin (101), but Adolf's (8) more recent results have led him to conclude that alkalies and alkaline earths dissolve serum globulin in equivalent proportions. Both proteins combine with bases in equivalent proportions after they are dissolved and more of the reactive groups in the molecule have dissociated.

Neutral salts dissolve globulins, and Hardy (101), (102) has considered the distribution of the components in systems containing globulins and neutral salts. The presence of more than one saturating body in such systems appears probable. Hardy and Mellanby (206) found that the amount of globulin dissolved by a neutral salt was dependent not only upon the nature and concentration of the salt, but upon the amount of

globulin present. Solubility of the globulin in the systems he studied was approximately proportional to the initial concentration of globulin. Sørensen (319) has recently confirmed the observations of Mellanby that the solubility of serum globulin in salt solutions is dependent upon the amount of the precipitate, and pointed out that such results can only be understood in terms of the phase rule, on the assumption that more than one saturating body is present. Sørensen has suggested that the saturating body in his experiments was a compound of pseudo- and euglobulin. Certain of these results might be explained on the older hypothesis that the precipitate was a mixture of the insoluble compounds of different globulin fractions with inorganic ions, but his new and in large part as yet unpublished experiments have led him to discard this theory. In either case a given concentration of a neutral salt would dissolve more of the more soluble globulin fraction, and the composition of the precipitate would progressively change, in the way that he has described.

The effect of neutral salts in dissolving globulins has been studied a number of times (10), (38), (101), (141), (194), (206), (247), (305), (319), but the nature of the phenomenon is not yet entirely clear. Many times as much salt, as acid or alkali, is required to dissolve a globulin (101), and solubility bears a logarithmic, rather than an arithmetic relation to concentration (52). The influence of the amount of the precipitate unquestionably indicates the existence of more than one saturating body. The added salt presumably combines with the protein (234), (238) and distributes itself between the precipitate and the solution, thus producing enormously complicated systems (38), (101), (319). Amino acids evidently behave in a comparable manner (274), (275), (276), (277), (278). Pfeiffer and his collaborators maintain that

> die Löslichkeit von Aminosäuren in Wasser in zahlreichen Fällen durch Neutralsalze stark erhöht. . . . Diese Loslichkeitserhöhung kann nur darauf beruhen, dass die Aminosäuren in wässriger Lösung mit den Neutralsalzen bzw. ihren Ionen Komplexverbindungen geben, von denen sich ja eine grössere Zahl in krystallisierter Form isolieren liess (275, p. 180).

The solvent action of neutral salts upon difficultly soluble compounds of different valence types has often been studied in recent years (33), (34), (35), (176), (236), and may offer an analogy to the behavior of globulins. Brønsted has studied a large number of metal ammonia compounds, and finds that neutral salts influence their solubility the more, the higher the valence of the solute and of the solvent. It is perhaps worth noting that serum globulin is dissolved by lower concen-

trations of salt than is edestin (101), (206), (247), and also that the square of the valence rule upon which solubility in non-protein systems depends, was first deduced from studies upon serum globulin. Mellanby wrote in 1905:

> Solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient, and the efficiencies of ions of different valencies are directly proportional to the squares of the valencies (206, p. 373).

The same rule was given in a more general form by Lewis and Randall in 1921 (175, p. 1112) and has found a theoretical explanation in the Debye-Hückel theory of interionic forces. There has as yet been no satisfactory demonstration, however, that the effects of neutral salts upon globulins, and upon other difficultly soluble substances, are more than analogous.

XII. The solubility of proteins in concentrated salt solutions. Following the observation that proteins could be precipitated by neutral salts, methods were developed by means of which proteins could be separated from each other. It was found that when a solution containing several proteins was brought to a critical concentration of a salt, the less soluble proteins were precipitated, while the more soluble remained in solution. Not only have proteins been found to differ in their solubility in the same salt solution, but different salts have been shown to have different precipitating action. Thus saturated solutions of certain salts have been found to precipitate some proteins, but not others. On the basis of these phenomena, a technique of "salting out" has been developed, by means of which the identity of a large number of proteins has been established. Upon this technique the separation and purification of many proteins depends, and, to a certain extent, their classification.

The nature of the phenomena involved in the "salting out" of proteins has in large part been revealed by recent quantitative studies upon purified proteins. In 1913 Chick and Martin published a "contribution to the theory of the salting-out of proteins" (45, p. 380). They studied "the precipitation of egg-albumin by ammonium sulfate," and found that the conditions that affected solubility in systems containing egg albumin and ammonium sulfate were (a), the concentration of salt; (b), the concentration of protein; and (c), the hydrogen ion concentration.

Effect of the protein concentration upon solubility. Chick and Martin "made an experiment with pure egg-albumin, estimating the protein

TABLE 11

The solubility of egg albumin in systems containing $(NH_4)_2\,SO_4$

Defined by the equation $\log s = \alpha m + \beta$

INVESTIGATORS	$(NH_4)_2SO_4$ CONCENTRATION	SOLUBILITY OF EGG ALBUMIN	LOG OF SOLUBILITY	$\frac{\log s_1 - \log s_2}{m_1 - m_2}$	$\log s - \alpha m$
	m†	*s: grams per liter*	$\log s$	$-\alpha$	β
Chick and Martin (45, p. 382)	1.920	17.4	1.2405*		
	2.029	11.45	1.0588	3.62	8.61
	2.134	4.90	0.6902	3.72	8.63
	2.250	1.72	0.2355	3.83	8.61
	2.510	0.43	$\bar{1}$.6335*		
Average				3.72	8.62
Chick and Martin (45, p. 383)	1.920	16.05	1.2054*		
	1.977	15.12	1.1796*		
	2.033	11.85	1.0738	3.60	8.68
	2.089	7.90	0.8976	3.91	8.71
	2.144	4.45	0.6484	3.70	8.67
	2.170	3.79	0.5786	3.58	8.69
	2.253	1.74	0.2405	3.90	8.67
	2.412	0.37	$\bar{1}$.5682*		
Average				3.74	8.68
Sørensen and Høyrup (323, p. 224)	1.612	21.22	1.3267	3.44	7.09
	1.638	17.35	1.2392	3.43	7.10
	1.674	13.61	1.1338	3.57	7.12
	1.704	10.45	1.0191	3.63	7.12
	1.735	7.87	0.8960	3.62	7.11
	1.770	5.93	0.7731	3.57	7.11
	1.828	3.83	0.5832	3.58	7.13
	1.878	2.45	0.3892	3.67	7.11
	1.928	1.53	0.1847	3.71	7.09
	1.986	0.99	$\bar{1}$.9956	3.58	7.10
Average				3.58	7.11

* Represented graphically but not employed in calculations.

† The concentrations in tables 11, 12 and 13, have been calculated by means of Sørensen's table (325, p. 158).

precipitated by a constant concentration of ammonium sulfate . . . . when the amount of protein was varied" (45, p. 385). They concluded from this experiment that "not only is more protein separated

from the more concentrated solution at a given concentration of salt, but a greater proportion is precipitated," when the protein concentration is increased. Their conclusion, that solubility in such systems was a function of the amount of the saturating body, seemed difficult to reconcile with classical chemistry on the basis of a two phase system. The explanation of the observations of Chick and Martin was furnished by Sørensen and Høyrup in 1917 (322). These investigators demonstrated by a "method of proportionality" that egg albumin was hydrated in solution. They have estimated that 1 gram of anhydrous egg albumin

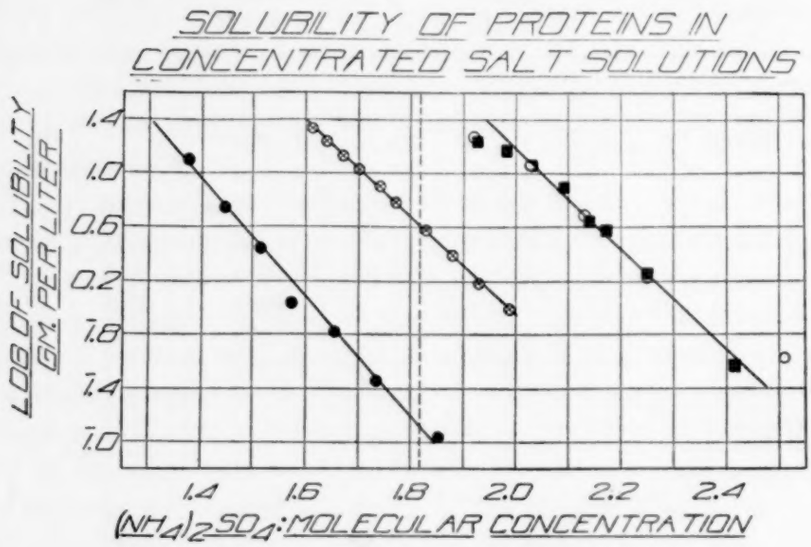


Fig. 7

Investigators		*Protein*	*Symbol*
Chick and Martin (45, Table 1)		Egg albumin	○
Chick and Martin (45, Table 2)		Egg albumin	■
Sørensen and Høyrup (323, Table 40)		Egg albumin	⊗
Sørensen (319)		Pseudoglobulin	●

The straight lines have been drawn through points calculated by means of equation (35), and the constants α and β in tables 11 and 12. The points represent experimental determinations. The dotted line designates half saturated ammonium sulfate.

bound 0.22 gram of water in their experiments. When they applied this correction to the solubility measurements of Chick and Martin, it appeared that the effect of the high concentrations of albumin had been to diminish the water in the aqueous phase, and thereby increase the ammonium sulfate concentration.

As a result of their recalculation, Sørensen and Høyrup believe that the differences in solubility observed by Chick and Martin "may be easily accounted for as an effect of the different salt concentrations of the filtrates" (323, p. 252). Experiments of their own, arranged "in such a way that the concentration of ammonium sulphate" was "really the same in all the filtrates," convinced them that "the state of equilibrium was independent of the protein concentration" (323, p. 252).

Effect of the salt concentration upon solubility. The effect of the concentration of ammonium sulfate upon the solubility of egg albumin has been studied both by Chick and Martin (45) and by Sørensen and Høyrup (323). The former noted, in a graphical representation of their two experiments in which the ammonium sulfate in the systems was plotted as abscissa and the albumin in the filtrate as ordinate, that "the point of commencing precipitation" was very sharp, and that "the curve then descends steeply and approaches the base line asymptotically" (45, p. 384).

The results of these two experiments, recalculated by the method to which Sørensen subjected the experiments of Chick and Martin that have already been referred to, are reported in the accompanying table. The comparable, though far more elaborate, investigation of Sørensen and Høyrup has been added. The "egg albumin hydrate" has been expressed as grams per liter, and the ammonium sulfate in molecular concentrations. In the graphical representation of the measurements made in these researches, instead of the solubility of the albumin, its logarithm has been plotted against the ammonium sulfate concentration. A straight line results. The consistency with which the experimental points fall upon the calculated straight line in figure 7 is evidence of the accuracy, both of the methods employed by the different investigators and of the implied relation.

The relation between the concentration of salt and the logarithm of the solubility furnishes a quantitative description of the "salting out" of proteins. The relation may be expressed by an equation of the type:

$$\log s = \alpha m + \beta. \tag{35}$$

In this equation s is the solubility of the protein, m the molecular concentration of the salt, α the proportionality factor, and β another constant. The slopes of the straight lines in figure 7 are measured by α, and their intercepts by β.

Observation suffices to show that the slopes of the straight lines passing through Chick and Martin's and Sørensen and Høyrup's

experimental points are very similar. The characteristic constants in the table have been calculated by solving the equation relating the solubilities s_1 and s_2 to their respective salt concentrations, m_1 and m_2:

$$\alpha = \frac{\log s_1 - \log s_2}{m_1 - m_2} \tag{36}$$

The average value of α derived from equating all the observations in each experiment against the others was 3.73 for Chick and Martin's measurements and 3.58 for Sørensen and Høyrup's. The agreement is very satisfactory. Sørensen and Palitzsch have also studied the precipitation of egg albumin by phosphates (328).

Since 1920 Sørensen has been studying serum globulin in much

TABLE 12

The solubility of pseudoglobulin in systems containing $(NH_4)_2SO_4$

Defined by the equation $\log s = \alpha m + \beta$

INVESTIGATOR	$(NH_4)_2SO_4$ CONCENTRATION	SOLUBILITY OF PSEUDOGLOBULIN	LOG OF SOLUBILITY	$\frac{\log s_1 - \log s_2}{m_1 - m_2}$	$\log s - \alpha m$
	m	*s: grams per liter*	$\log s$	$-\alpha$	β
Sørensen (319, p. 469)*	1.374	12.54	1.0983	4.64	7.14
	1.448	5.45	0.7364	4.54	7.11
	1.514	2.74	0.4378	4.52	7.10
	1.575	1.38	0.1399	4.45	7.07
	1.654	0.66	$\bar{1}$.8195	4.33	7.10
	1.733	0.29	$\bar{1}$.4624	4.31	7.09
	1.851	0.11	$\bar{1}$.0414	4.03	7.19
Average				4.40	7.11

* We are indebted to S. P. L. Sørensen for supplying the data upon which his curve and these calculations are based.

the same manner as he had previously studied egg albumin. The first report of these investigations has just appeared (319), and includes a curve relating the solubility of the pseudoglobulin fraction studied, expressed as grams "globulin hydrate" to grams ammonium sulfate. His experimental points have also been plotted in figure 7. They fall upon a straight line, as did his egg albumin solubilities, if the logarithm of solubility is plotted against the molecular concentration, suggesting that the relations that have been discussed may be of general application. The slope of the line is different, however, from the slope of the egg albumin. The calculated constant α has a value of approximately

4.40. Presumably "the application of this equation to the precipitation of proteins by neutral salts offers a method of characterizing very soluble proteins" (52, p. vii).

Effect of the hydrogen ion concentration upon solubility. Although the constant α, as determined from the observations of Chick and Martin upon egg albumin, was of the same order as that deduced from Sørensen and Høyrup's more accurate measurements, the values for β, the other constant in equation (35), were very different. This may depend upon

TABLE 13

The solubility of egg albumin in systems containing $(NH_4)_2SO_4$ and varying amounts of NH_4OH

Defined by the equation $\log s = \alpha m + \beta$

INVESTIGATORS	$(NH_4)SO_4$ CONCENTRATION	ALBUMIN HYDRATE IN SYSTEM	SOLUBILITY OF ALBUMIN HYDRATE	LOG OF SOLUBILITY	$\log s - \alpha m$	HYDROGEN ION CONCENTRATION
	m	*grams per liter*	*s: grams per liter*	$\log s$	β	pH^+
Sørensen and Høyrup (323, p. 244)	1.813	86.42	1.97	0.2945	6.78	4.54
Sørensen and Høyrup "	1.743	85.94	3.89	0.5899	6.82	4.50
Sørensen and Høyrup "	1.813	86.42	2.02	0.3054	6.80	4.64
Sørensen and Høyrup "	1.743	85.94	4.02	0.6042	6.84	4.65
Chick and Martin (45, p. 388)	1.90	11.10	0.77	$\bar{1}$.8865	6.97	4.69
Sørensen and Høyrup (323, p. 244)	1.813	86.42	3.01	0.4786	6.97	4.85
Sørensen and Høyrup "	1.743	85.94	5.99	0.7774	7.02	4.85
Chick and Martin (45, p. 388)	1.98	10.44	0.68	$\bar{1}$.8325	7.22	4.98
Sørensen and Høyrup (323, p. 244)	1.813	86.42	5.28	0.7226	7.21	5.00
Sørensen and Høyrup "	1.743	85.94	9.82	0.9921	7.23	4.98
Chick and Martin (45, p. 388)	1.90	11.10	2.12	0.3263	7.41	4.90
Sørensen and Høyrup (323, p. 244)	1.813	86.42	15.95	1.2028	7.69	5.22
Sørensen and Høyrup "	1.743	85.94	29.14	1.4645	7.70	5.30
Chick and Martin (45, p. 388)	1.90	11.10	6.50	0.8129	7.90	5.46
Chick and Martin "	1.98	10.44	6.27	0.7973	8.18	5.40

the fact that, whereas Sørensen and Høyrup's experiments were conducted at the isoelectric point, Chick and Martin's probably were not, for the hydrogen ion concentrations of their systems were not reported.

Nevertheless, Chick and Martin understood the significance of the hydrogen ion concentration, and investigated its effect upon the precipitation of egg albumin by ammonium sulfate. Two of their experiments are in fair agreement with each other, and with a comparable experiment performed by Sørensen and Høyrup. The measurements of these investigators upon the solubility of albumin in different concen-

trations of salt and at different hydrogen ion concentrations are reported in table 13. From them and from the values of α deduced from their experiments, values of β have been estimated by means of equation (35) on the assumption that, as a first approximation, α can be considered constant over a narrow range of hydrogen ion concentrations. It follows from the equation that in so far as α is a true constant, β must be independent of the salt concentration. Theoretically, therefore, β yields the solubility of a salt free albumin solution. Despite a wide variation in the concentration of ammonium sulfate, and an eight-fold

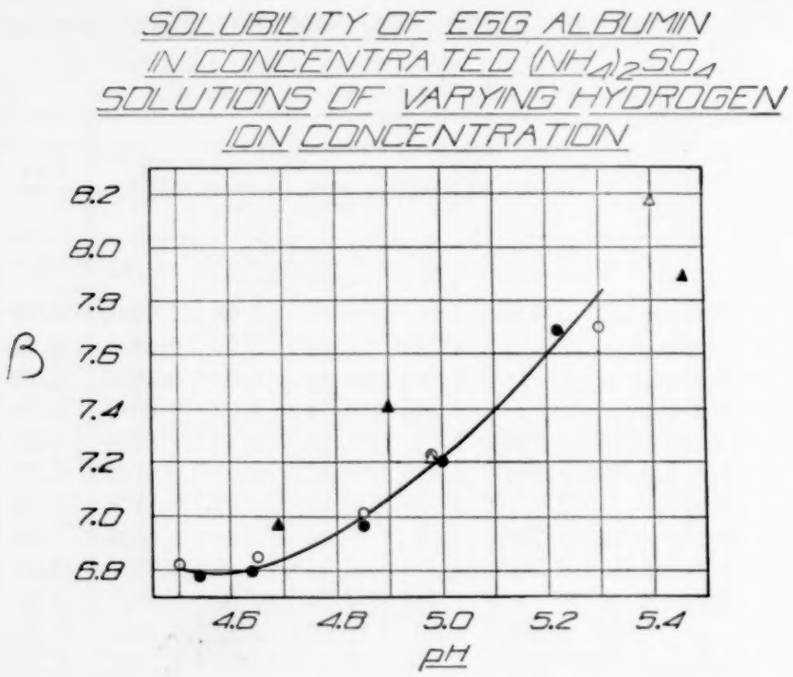


Fig. 8

Investigators	$(NH_4)_2SO_4$:*m*	*Symbol*
Sørensen and Høyrup (323, Table 44)	1.743	○
Sørensen and Høyrup (323, Table 44)	1.813	●
Chick and Martin (45, Table 6)	1.90	▲
Chick and Martin (45, Table 5)	1.98	△

variation in the albumin in the systems, the calculations in table 13 reveal the fact that at any one hydrogen ion concentration, β was constant within the experimental error, and that β varied as a function of the hydrogen ion concentration. This relation is represented graphically in figure 8.

In all probability the increase in β, as the hydrogen ion concentration becomes smaller than the isoelectric reaction, depends upon the dissocia-

tion of the acid groups of egg albumin in precisely this range. These ammonium albumin compounds are presumably more soluble than the ammonium sulfate compounds, whose existence in the neighborhood of the isoelectric point Sørensen and his collaborators have demonstrated (323). Accordingly solubility is increased by their presence. It should, however, be possible to calculate the amount of this soluble compound from the dissociation constants of the groups involved, and the hydrogen ion concentration. In the absence of such groups, β must assume a minimal value β_0, smaller than 6.8 for egg albumin.

The constant β is thus constituted of two terms, of which the one increases as the protein forms more soluble compounds, while the other β_0 represents the solubility in water of the soluble protein compound in the absence of salt. This follows from putting m equal to 0 in equation (35). The log of the solubility in the absence of salt, which may be called s_0, then becomes equal to β_0. The very high concentrations of salt in which the protein can alone be studied in these cases renders extrapolation to salt free conditions impossible. It may be noted, however, that the solubility in water of the hypothetical albumin compound in question would be of the order of 10^6 grams per liter. These figures become scarcely less fantastic when transformed into molecular concentrations, when they assume the order of magnitude of 10.[2]

Although Sørensen has not yet published experiments relating the solubility of globulins in salt solutions to the hydrogen ion concentration, and no estimate of β_0 can therefore be made, it is probable that the s_0 value of this globulin compound, though much lower than the s_0 value of the comparable albumin compound, must be of the same order of magnitude. The difference in the solubility of this compound and of the pure globulin in water is very striking, and suggests the great solvent action of small quantities of salt upon globulins.

The separation of proteins from each other by "salting out" depends at least in part upon differences in the values of β. This appears from the consideration of the β values that have been derived for egg albumin and pseudoglobulin. Presumably the pseudoglobulin that Sørensen studied was at, or near, its isoelectric point, as was his egg albumin preparation. The respective β values are 7.11 and 7.11. Although there is no doubt, therefore, that the albumin is more soluble than the pseudoglobulin, inspection of figure 7 indicates that these proteins could not be quantitatively separated from each other, if they had the same isoelectric point. Presumably Chick and Martin studied the precipitation of egg albumin at a hydrogen ion concentration in the

neighborhood of the isoelectric point of the globulin. Here an almost quantitative separation could be effected, since the β value of the egg albumin, 8.66, is much greater than that of the globulin, namely, 7.11. Clearly the separation of proteins from each other by "salting out" depends not only upon their respective solubilities, but also upon their respective isoelectric points, and therefore upon the hydrogen ion concentration.[17]

Significance of the equation defining solubility in strong salt solutions. The type of equation that had been deduced from a consideration of the solubility studies upon egg albumin and serum globulin of Chick and Martin, and of Sørensen and his collaborators, has a theoretical significance.

> "When, at a given temperature, a solid salt is in equilibrium with a solution, the activity of that salt in the solution is fixed. It cannot be changed by any change in the nature of the solution, such as would be produced by the addition of other electrolytes. . . . . Since . . . . the activity coefficient is defined as $\gamma = a_{\pm}/m_{\pm}$, we see that whatever happens (isothermally) to the solution, the activity coefficient of the salt in question must remain inversely proportional to the mean molality of its ions" (176, p. 369)

and therefore to its solubility. Solubility is thus a measure of the ratio of the activity of the ion to its concentration (33), (176), (236).

Wilson (358) has employed an equation identical to (35) in characterizing the measurements of Thomas and Baldwin (338) upon the effects of alkali chlorides upon the hydrogen potential, and Linderstrøm-Lang (182), working in Sørensen's laboratory, has similarly described the effects of neutral salts upon the potential of the quinone-hydroquinone electrode. Debye and McAuley have considered the effects of neutral salts upon sugar solutions. The equation that they employ differs from equation (35) only in that they have expressed the constant α in terms of the mean radius of the ions, the ionic strength, and the dielectric constant of the solution. The precipitating action of neutral salts upon proteins thus appears to depend upon the charge and the dimensions of the ions, both of the protein and of the neutral salt. In these terms an explanation may therefore be sought, both of the order

[17] The studies of Loeb and Kunitz (164), (196), on the effect of salts upon other properties of protein solutions did not reveal the Hofmeister series. Their salt solutions were, however, more dilute than those required to effect precipitation. In dilute solutions the dimensions of the ions become negligible in comparison with the distance between them, and under these conditions the valency of ions seems to be the most important factor in determining their behavior.

in which Hofmeister (137) and subsequent investigators[17] (145), (178), (181), (330) have described the precipitating power of different ions upon the same protein, and of the order in which different proteins are precipitated by the same neutral salt (143), (146), (156), (246). The serum proteins are precipitated in the order: euglobulin, pseudoglobulin, serum albumin, and this is the order of their molecular weights[18] and probably of their molecular volumes.

XIII. THE VISCOSITY OF PROTEIN SOLUTIONS. Proteins differ greatly from each other with respect to the viscosity of their solutions. Moreover, the viscosity of the same protein varies, not only with change in protein concentration, but also with change in hydrogen ion concentration and with salt concentration. For these reasons the viscosity of a protein solution has often been employed as a method of defining its physicochemical state.

Relation between the physicochemical state of a protein solution and its viscosity. The effects of acids, bases, and salts upon the viscosity of protein solutions were so carefully noted by Sackur in 1902[19] that the task of succeeding investigators has been to supplement and to interpret his observations. Sackur made measurements upon solutions of sodium caseinate and observed that the viscosity of a protein solution varied with the amount of base with which it was combined; viscosity was increased by the addition of a small amount of base, and decreased by the addition of more base. Moreover he noted that neutral salts further diminished viscosity. Sackur accordingly correlated these changes in the viscosity of his casein solutions, with changes in dissociation.

The hypothesis, that changes in the viscosity of protein solutions were due to changes in their dissociation, has influenced all subsequent work. Hardy (101), who next investigated the viscosity of protein solutions, planned his experiments to test Sackur's theory. He argued that, if Sackur's views were correct, globulins should have a relatively low viscosity when dissolved in neutral salt solutions, "since their solution in neutral salts contains no ionic, no electrically charged globulin, as is shown by the absence of all movement in an electric field, while solutions in dilute acids and alkalies always are 'ionic'" (101, p. 284). His experiments demonstrated that "the viscosity of solutions of alkali

[18] By inference the molecular volume, and probably the molecular weight of fibrin is greater than euglobulin. Since its minimal molecular weight is 14,000 or 42,000, its molecular weight may be in the neighborhood of 168,000.

[19] An earlier paper by Bottazzi has not been available for study.

globulin and of acid globulin is much higher than that of solutions of salt globulin of the same strength and of the same grade." Accordingly Hardy concluded that "the presence of ionic globulin in solution raises the viscosity very much" (101, p. 284, and that "excess of alkali diminishes the viscosity . . . . no doubt partly due to the free alkali or acid depressing the degree of ionisation of the globulin salt and so far it confirms Sackur's view that the high viscosity is due to the proteid ions" (101, p. 285).

The more extensive invesigations of Pauli and his co-workers (263, (264), (266), (271), (272), (304), and of Loeb (192), (193), (195), upon the variation in the viscosity of protein solutions with change in hydrogen ion concentration on the whole confirm the observations of Sackur and Hardy. Moreover Pauli and his co-workers, and later Loeb, demonstrated that the viscosity minimum, like the precipitation maximum, coincided approximately with the isoelectric point.

Different investigators have thus described the changes in the viscosity of protein solutions brought about by acids and alkalis in very much the same way. They have, however, suggested different mechanisms than those suggested by Sackur in explanation of the phenomena. The charges upon colloids rather than the dissociation of electrolytes were responsible for Hardy's view that "the density of the electric charge if the internal surfaces be electrified . . . . will decrease the fluidity owing to the fact that electric work must be done in deforming the solution" (101, p. 282). Pauli believed that the lower viscosity of neutral than of ionized protein was due to its lower "hydration or imbibition" (263, p. 37). Chick and her collaborators also considered that the proteins were hydrated in the solutions whose viscosity they measured (39), and the amount of water that appeared to be bound in their experiments has been variously estimated by Arrhenius (15), (16), and by Hatschek (113).

The mechanism in terms of which Loeb explained the higher viscosity of ionized than of neutral protein also involved a change in volume. He believed that this was brought about, not by hydration, but by swelling due to osmotic pressure. This swelling he related to that involved in the Donnan equilibrium. The experiments which led him to this conclusion were performed upon egg albumin and gelatin. He observed, as had Chick and Lubrzynska (41), that egg albumin behaves very differently from a more viscous protein:

> the difference in the order of magnitude of the viscosity of the two proteins must be due to the fact that gelatin possesses a mechanism for increasing its relative

volume in solution which is lacking in the case of egg albumin . . . . and this mechanism seems to be connected, in the case of gelatin solutions, with their tendency to set to a gel. . . . . If we adopt this idea for the explanation of the high order of viscosity of gelatin solutions as compared with solutions of egg albumin, we reach, the conclusion that the gelatin solutions contain submicroscopic particles of solid jelly. . . . . There must arise a Donnan equilibrium between these submicroscopic particles of solid jelly and the surrounding solution, and this Donnan equilibrium must regulate the amount of water occluded by the submicroscopic particles of solid jelly, floating in the gelatin solution. Since the low order of magnitude of the viscosity of albumin solutions excludes the existence of a considerable number of such submicroscopic particles in the solution, it becomes obvious that the Donnan equilibrium cannot manifest itself to any large extent in the viscosity of solutions of this protein (195, first edition p. 204–206).

The apparent obliteration of the influence of the hydrogen ion concentration upon the viscosity of egg albumin of low concentration that Loeb observed had been noted by Hardy in the case of the more viscous protein, serum globulin. Wide variations in the concentration of acid or of alkali added to 0.62 per cent solution of this protein changed the relative viscosity only from 1.17 to 1.31. As a result Hardy concluded that "dilution diminishes the difference between the various solutions" (101, p. 285). The influence of the hydrogen ion concentration upon the viscosity of protein solutions is thus seen to be related to the protein concentration.

Relation between the concentration of a protein solution and its viscosity. Certain theoretical considerations led Einstein (78) to deduce an equation for the viscosity of a suspension or solution in which "the dissolved particles are of spherical form and of great magnitude compared with the molecules of the solvent" (16, p. 112). In this equation:

$$\eta = \eta_0 (1 + 2.5 \varphi) \qquad (37)$$

"η is the viscosity of a solution containing the volume fraction φ of a dissolved substance in unit volume of a solvent with the viscosity η_0 at the same temperature" (16, p. 112). Although Einstein assumed spherical, non-compressible, and non-hydrated particles, the other assumptions underlying this equation suggested that it might be applicable to protein solutions. Lewites (177), and later Loeb (192), employed it in characterizing dilute gelatin solutions, and Arrhenius (16) and Loeb (193) have shown that it applies to solutions of egg albumin and serum albumin. Arrhenius has calculated that it yields the constants for these two proteins as 2.95 and 2.14 respectively. Al-

though the theoretical value of this constant is 2.5, the agreement in these cases may be considered satisfactory. The Einstein equation does not define the relation between the concentration and the viscosity of the other proteins that have been examined, however, and the constants estimated from their viscosities in dilute solution achieve values that were not contemplated by Einstein's theory.

An empirical equation relating the change in viscosity with concentration of a large number of electrolytes and non-electrolytes was developed by Arrhenius in 1887 (13). According to this equation "the viscosity η of an aqueous solution of the concentration c ought to be subject to the condition

$$\log \eta = \theta c, \tag{38}$$

where θ is a constant and the viscosity of water at the same temperature is put equal to unity" (16, p. 115). Sackur (303) and later Lewites (177) employed this formula in the calculation of their results, and found that it held admirably for proteins, although the viscosities they observed were far greater than those of the solutions from which Arrhenius had deduced this logarithmic formula.

Some ten years after Sackur had measured the viscosity of a solution of sodium caseinate, Chick and Martin made measurements upon the same protein. This was the first of a series of investigations made by Chick and her collaborators that remain the most extensive and the most accurate measurements of the viscosities of protein solutions that have been reported.

Arrhenius analyzed the viscosity measurements of Chick and her collaborators in terms of his logarithmic formula, employing "two arbitrary constants in the formula . . . namely not only the constant θ but also the quantity n of water bound to one gram of protein substance for the calculation of the molecular concentration" (16, p. 117). The concentrations that Arrhenius employed were, of course, not molecular concentrations. "For substances like proteins, whose molecules are extremely large compared with those of water, he considers that c is expressed with sufficient accuracy by the equation.

$$c = \frac{100p}{100 - (n+1)p}, \tag{39}$$

in which p is the number of g. of solute in 100 g. of solution, and n the hydration factor i.e., the number of g. of solvent associated with

each g. of solute, and therefore withdrawn from the free solvent. The complete expression for the logarithm of the viscosity of a solution in which the molecular weight of the solute is high compared with that of the solvent, is accordingly" (113, p. 325):

$$\log \eta = \theta \frac{100p}{100 - (n+1)p} \tag{40}$$

Loeb studied the viscosity of egg albumin at 15°C. and of gelatin solutions at different temperatures (193). Like Sackur and Lewites, he employed the Arrhenius equation in characterizing his experiments. Numerous other investigators have studied the viscosity of one or another protein under various physicochemical conditions. The constants θ and n calculated from certain of their measurements appear in table 14, together with those that Arrhenius deduced from the measurements of Chick and her collaborators. The curves in the accompanying figure have been constructed from some of these constants. The consistency with which the experimental points fall upon the curves suggests that even if Arrhenius' equation is only considered "a purely analytical interpolation formula" (113, p. 330), it is capable of accurately representing the viscosities of protein solutions of different concentrations.

Relation between the viscosity of a solution containing several proteins and the viscosities of the several proteins. Viscosity is a colligative property,[20] and it should therefore be possible to analyze the viscosity of a solution containing several proteins in terms of viscosities due to the several protein molecules. The logarithm of the viscosity of a solution is equal to the sum of the products of the logarithms of the viscosities of the dissolved molecules and their respective volumes (15).

$$\log\eta = v_1 \log\eta_1 + v_2 \log\eta_2 + v_3 \log\eta_3 = \theta_1 c_1 + \theta_2 c_2 + \theta_3 c_3 \tag{41}$$

Chick and her collaborators studied the viscosity of whole serum as well as of serum albumin, pseudoglobulin, and euglobulin. The horse serum that they used contained 7.31 per cent of protein, and had a coefficient of viscosity of 1.72 (41). At this concentration, log η was therefore 0.236. On the assumption that no association occurs between the proteins in serum, it should be possible to calculate this viscosity from the sum of the viscosities of the constituent proteins. This cannot be done accurately at the present time, because serum albumin, pseudo-

[20] Lees (169) believes that fluidity rather than viscosity is the colligative property.

TABLE 14

The viscosity constants of certain protein solutions

PROTEIN		°C.	n	θ	PROBABLE MOLECULAR WEIGHT
Egg albumin; isoelectric	(193, p. 81)	15	0.5	0.0062	33,800
pH not reported	(41, p. 62)	42	1.0	0.0146	
" "	"	33	1.0	0.0152	
" "	"	25	1.0	0.0159	
" "	"	15	1.0	0.0165	
" "	"	8	1.0	0.0167	
" "	"	0	1.0*	0.0172*	
" "	(41, p. 60)	25	0.7*	0.0184*	
Serum albumin; isoelectric	(†)	25	1.3	0.0194	45,000
" pH not reported	(41, p. 65)	25	1.5*	0.0207*	
Hemoglobin; isoelectric	(†)	25	0.5	0.0143	50,000
Pseudoglobulin; isoelectric	(†)	25		0.0310	81,000
" basic compound	(39, p. 269)	25	1.2*	0.0402*	
Euglobulin; isoelectric	(39, p. 267)	25	2.2*	0.0625*	135,000
" basic compound	(39, p. 264)	25	1.8	0.0663	
Gelatin; isoelectric	(193, p. 84)	60		0.042	
" "	"	45		0.053	
" "	"	35		0.060	
" "	"	25		(0.063)	
" "	(67, p. 1536)	25		0.07	
" basic compound	(26, **)	35		0.12	
	(177, p. 210)	35		0.13	
" "	"	35		0.16	
" "	(237, p. 706)	40		0.134	
Casein; basic compound	(43, p. 103)	25	1.0*	0.113*	192,000
" "	(255, p. 348	20		0.12	
" "	(303, p. 677)	15		0.128	

* Estimates of n, and θ, made by Arrhenius (16).

† Unpublished measurements made by R. E. L.Berggren in this laboratory.

** We are indebted to R. H. Bogue for supplying the data upon which his previous calculations and those here reported have been based.

globulin, and euglobulin have not been studied under the conditions that obtain in the serum. The values of θ and of n derived from measurements of the viscosities of these proteins in other physicochemical states

can, therefore, only be employed in the following calculations to illustrate the principle involved. The distribution of the protein fractions

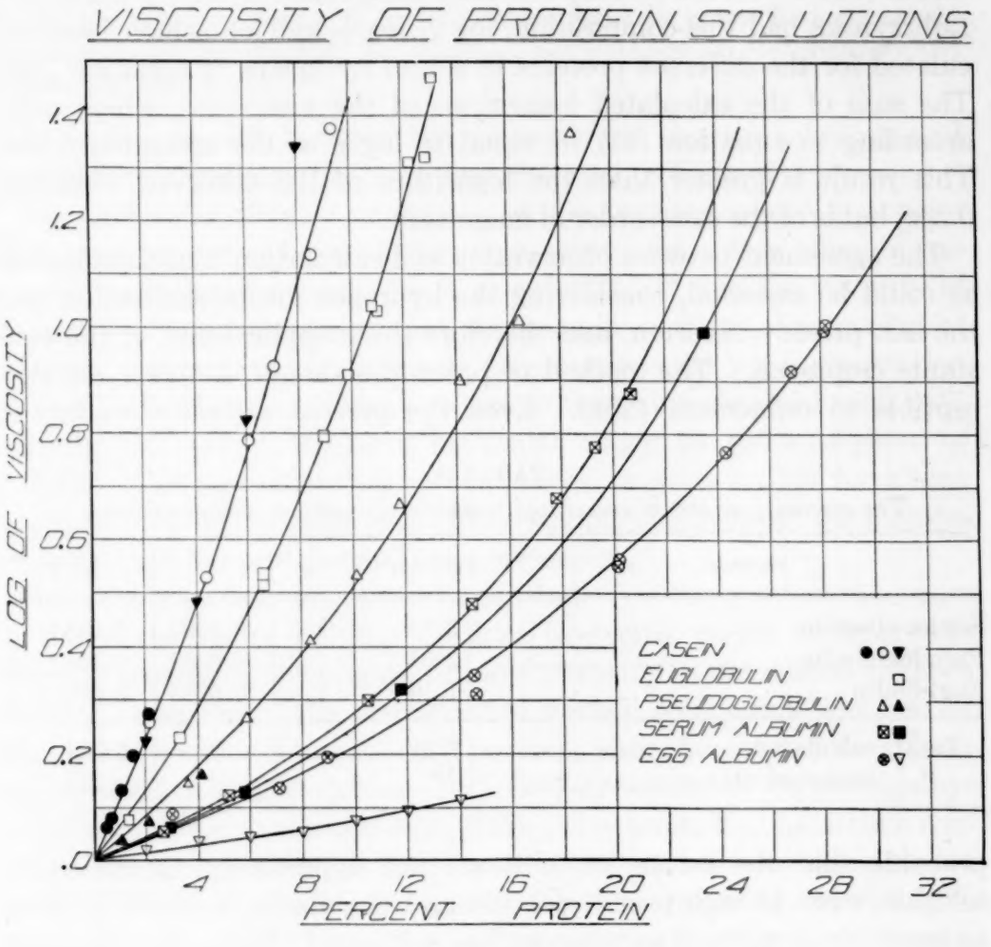


Fig 9

Investigators	*Protein*	*Symbol*
Sackur (303)	Casein	●
Chick and Martin (43)	Casein	○
Palitzsch (255)	Casein	▼
Chick (39)	Euglobulin	□
Chick (39)	Pseudoglobulin	△
Cohn and Berggren[3]	Pseudoglobulin	▲
Chick and Lubrzynska (41)	Serum albumin	⊠
Cohn and Berggren[3]	Serum albumin	■
Chick and Lybryzynska (41)	Egg albumin	⊗
Loeb (193)	Egg albumin	▽

varies considerably in different sera. The average figures for normal horse serum given by Bächer and Kosian (18) are 52.5 per cent albumin,

32.5 per cent pseudoglobulin, and 15.0 per cent euglobulin. These results are in good agreement with the measurements of Howe upon calf serum (144). On the assumption that the serum studied by Chick and Lubrzynska had this composition, log η_1, log η_2, and log η_3 have been calculated for the different proteins in serum by means of equation (40). The sum of the calculated logarithms of the viscosities, which must according to equation (41) be equal to log η of the serum, is 0.264. This result is greater than the logarithm of the observed viscosity, 0.236, but is of the same order of magnitude.

The agreement between observation and calculation is as satisfactory as could be expected, considering the hydrogen ion concentration and the salt present in serum, and therefore the inapplicability of the constants employed. The method of procedure should, however, be susceptible to refinement (301). Even the present estimate renders it

TABLE 15

The viscosity of serum calculated from the viscosity of serum proteins

PROTEIN	PER CENT	n	θ	log η
Serum albumin	3.84	1.5	0.0207	0.088
Pseudoglobulin	2.37	1.2	0.0402	0.101
Euglobulin	1.10	1.8	0.0663	0.075
Total, calculated	7.31			0.264
" observed (41)	"			0.236

probable that the serum proteins are not appreciably associated in solution, even though pseudoglobulin and euglobulin combine to form an insoluble compound as Sørensen has suggested (319). For the presence in serum of a large compound molecule of pseudoglobulin and euglobulin should still further emphasize the "disproportionate influence upon the viscosity of the whole" "that the small proportion of 'euglobulin' contained in the serum exercises" (41, p. 68). There would then result a still higher viscosity, since, as we shall see, the viscosity of a protein appears to be greater, the greater its molecular volume.

Relation between molecular weights of proteins and the viscosities of their solutions. In considering the viscosities of certain esters, Arrhenius noted that his constant θ appeared to be greater for the acetates than for the formates, and to increase in each of these groups with the molecular weight, and that it increases very rapidly with increasing molecular weight in the lower alcohol series (13, p. 291). Sackur was aware

of such a relation, as may be seen from his allusion to the work of Reyher (291).

Reyher hat gefunden, dass z. B. in der Fettsäurereihe die innere Reibung sowohl der freien Säuren wie die ihrer Natriumsalze mit wachsendem Molekulargewicht grösser wird (303, p. 677).

A similar relation between the viscosities of dextrin solutions and their molecular weights has been demonstrated by Biltz (21).

The molecular weights of certain of the proteins whose viscosities have been measured have already been considered. Egg albumin has a very low molecular weight for a protein, 33,800, and the viscosity of its solution "approximates to that obtaining in solutions of non-colloidal material"; whereas casein, which at " a comparatively low concentration (7 to 8%) produced a viscosity equal to the value obtained with 28% egg albumin" (41, p. 60) has a molecular weight six times as great, or 192,000. The molecular weights of the other proteins studied have been added to table 14, in order to facilitate comparison between the molecular weights of the proteins and their viscosities.

Such a comparison has led us to conclude that the greatest difference in the viscosities of proteins at their isoelectric points is dependent upon differences in the weights, or more probably in the related volumes of their molecules. The viscosities of protein compounds are greater than the viscosities of uncombined proteins, either because of the greater surface density of their charges (101), (303) or because of the accompanying change in their volumes (263), (195). The results that have been considered render it certain that the viscosities of the protein solutions are not independent of their molecular volumes, as they should be, according to Einstein's equation (37). This equation should hold for non-compressible, non-hydrated particles, but these assumptions render it inapplicable to proteins. Proteins are compressible (121), (255) and hydrated, and the electrically charged condition of their molecules greatly influences their viscosity. The evidence that is accumulating suggests that the viscosity of proteins increases both with the charge and with the size of their molecules.

The viscosity of a protein, like its crystallizability and its solubility, depends upon the dimensions and upon the charged condition of its molecules. The smaller proteins which contain the largest number of dissociable groups appear to be both crystallizable and soluble. Egg and serum albumin have the smallest molecules of the proteins that have been adequately investigated. They have low viscosities, are

very soluble in water, and are precipitated in crystalline form only from very concentrated salt solutions. Hemoglobin and edestin, which have heavier molecules,[21] are only slightly soluble in water at their isoelectric points, and may be crystallized there by dialyzing and by concentrating their solutions. Their solubility under these conditions is increased by salt, for which reason they are classified as globulins. The larger globulins of serum, although they are soluble in salt solutions, have not been crystallized, neither have gelatin nor casein, the largest proteins whose molecular weights have been estimated. These proteins are only slightly soluble when uncombined with acids or bases. Their compounds are very viscous and are precipitated by low concentrations of neutral salts. Thus the proteins that are small and of high valence seem to be readily crystallizable, soluble, and of low viscosity; whereas those that are large are very viscous, are only slightly soluble when uncombined with acids or bases, and have not yet been crystallized.

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[21] The same protein is more viscous when more dissociated, and the more dissociated of two proteins of approximately the same molecular weight also seems to be the more viscous. The molecular weight of hemoglobin is probably greater than that of serum albumin but its viscosity is lower. This manifests itself not only in a lower value of θ, but also of n. If n represents water of hydration, as Arrhenius believes that it does, the effective molecular volume of serum albumin as judged by viscosity measurements may be greater than that of hemoglobin. More accurate methods of estimating molecular volumes are required in order that this quantity may be correlated with viscosity and the other properties of proteins which appear to depend upon their dimensions.

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